THE CLONING AND CHARACTERIZATION OF THE *CLOSTRIDIUM PERFRINGENS* TYPE A 8-6 ENTEROTOXIN GENE

By

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This dissertation is dedicated to my mother, Delores, who shared with me her voracious reading habit which helped me to reach my goals

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Clostridium perfringens is one of the most ubiquitous and extensively studied bacterial pathogens. The virulence of this organism is due to its remarkable ability to produce a variety of different toxins, including the type A enterotoxin (CPE-A), which is the cause of a common human foodborne illness.

In 1985, a unique *C. perfringens* enterotoxin (CPE 8-6) was isolated from a mutant designated 8-6. The CPE 8-6 is similar to the CPE-A in molecular weight; however, it differs from the CPE-A both biochemically and in biological activity. The CPE 8-6, like the CPE-A, is superantigenic. However, it is not known if the superantigenic activity is linked to the enterotoxigenic activity in either of these two toxins. Both the protein and nucleic acid sequences for the CPE-A are known; however, the CPE 8-6 has not been similarly characterized.

The overall goal of this dissertation was to compare and contrast the CPE 8-6 to that of the CPE-A. *C. perfringens* type A and type A 8-6 strains were screened by ELISA, Western immunoblot, Vero cell cytotoxicity, and PCR analysis to determine enterotoxigenic activity. Restriction enzyme profiles between the genomic DNA from type A and type A 8-6 strains were compared using a series of DNA probes to identify differences; no differences were observed. The chromosomal location of the *cpeA* and *cpe86* genes were also determined.

The *cpe86* gene was cloned and sequenced, and the nucleic acid sequence and its upstream region was found to be the same as for the *cpeA* gene. This result was unexpected since the CPE 8-6 exhibits different characteristics than the CPE-A. To examine the possibility that the CPE 8-6 protein differs from the CPE-A in secondary or tertiary structure due to alterations in post-translational modification, matrix-assisted laser desorption ionization mass spectrometry was used. Preliminary results suggest that the CPE 8-6 is indeed modified differently from that of the CPE-A. This observation could account for the biological and biochemical differences observed between the two enterotoxins.

CHAPTER 1
INTRODUCTION

Species from the genera *Clostridium*, *Escherichia*, *Staphylococcus*, *Streptococcus*, and *Campylobacter* are well known bacterial pathogens and are responsible for most of the foodborne illnesses in the USA. In the last ten years these microorganisms have been found to affect the human host in a more complex manner than the classical foodborne illness symptoms of diarrhea and emesis. For example, they appear to have a significant role in Reiter's syndrome, rheumatoid arthritis, hemolytic uremic syndrome, Guillain-Barre syndrome, toxic shock syndrome, Kawasaki disease, Lupus-like disease, rheumatic heart disease, infant botulism, and sudden infant death syndrome (SIDS) (Bunning, 1994; Lindsay *et al.*, 1994). Therefore, there has been an increased interest in the mechanisms and expression of pathogenicity of these organisms. As a result, it was discovered that many bacterial virulence factors can actually modulate the host defense system by affecting certain lymphoid cell populations and that these factors may be important in the overall pathogenicity of the organism. Perhaps the most important finding was that some virulence factors have superantigenic properties. That is, they can stimulate as many as 1 in 5 T lymphocytes in contrast to a classical antigen that stimulates only 1 in 10,000 T lymphocytes. The effect of this overstimulation is the induction of increased levels of cytokines such as interferons, tumor necrosis factor, and interleukins, which can lead to decreased blood pressure, shock, respiratory interference, multiple organ failure, and death (Kotzin *et al.*, 1993).

Most of the work on superantigens has been done on staphylococcal enterotoxins; however, other superantigens have been identified: streptococcal toxic shock syndrome toxin (TSST-1), *Streptococcus pyogenes* exotoxins, *Pseudomonas aeruginosa* exotoxin A, *Yersinia enterocolitica* antigen, and the *C. perfringens* enterotoxins (Fleischer, 1994). While the effect of superantigens on the immune system has been extensively studied, their functional role in virulence is unclear. In the future, the specific mechanisms by which superantigens contribute to disease should become more clearly defined, allowing for new therapies and treatment of some diseases.

Clostridium perfringens is possibly the most extensively studied anaerobic bacterial pathogen (McClane, 1995; Labbe, 1989; Hathaway, 1990). The organism is ubiquitous, occurring in soil, water, sewage, and the intestinal tract of animals and many humans. *C. perfringens* strains produce a variety of protein toxins which play an important role in the pathogenicity of the organism.

C. perfringens is associated with many human diseases such as foodborne illness, myonecrosis (gas gangrene), necrotizing enterocolitis of infants, antibiotic associated diarrhea, enteritis necroticans, acute sporadic diarrhea, and sudden infant death syndrome (SIDS) (Lindsay *et al.*, 1994; Labbe, 1989; Murrell, 1989). *C. perfringens* is also the causative agent of several enterotoxemic diseases and sudden death in animals.

C. perfringens is associated with two diseases that are transmitted by food. Type C strains are associated with a severe but rare food poisoning, necrotizing enteritis. Outbreaks occurred in Germany after World War II involving canned meats, and until recently the disease "pigbel" was endemic in New Guinea due to the consumption of undercooked pork (Labbe, 1989; Murrell, 1989). Type A strains can produce an enterotoxin (CPE-A) which causes the classical, mild form of food poisoning which is seldom fatal. The illness is quite common, accounting for 11-12% of all bacterial foodborne illness (FBI) in the USA. This superantigenic enterotoxin is primarily associated with FBI; however, recent evidence has indicated an association with SIDS (Lindsay *et al.,* 1993, 1994). Therefore, the enterotoxin may have a greater significance in human disease than previously thought.

During the last ten years, there has been considerable argument as to whether *C. perfringens* type A strains produce more than one type of enterotoxin. The type A enterotoxin (CPE-A) from strain 8239 is well characterized (McClane, 1994); however, in 1985 Lindsay *et al.* isolated another type A enterotoxin which appeared unique. This enterotoxin, CPE 8-6, was derived from a spontaneous mutant of strain NCTC 8798 designated 8-6. It was isolated by Duncan *et al.* (1972) during a classic experiment that first linked enterotoxin production to sporulation. The two enterotoxins have similar molecular weights, but appear biochemically and biologically different. CPE 8-6 has been shown to have 2 subunits with isoelectric points of 3.8 and 4.3 (Lindsay *et al.*, 1985), while CPE-A is composed of a single polypeptide (Czeczulin *et al.*, 1993) with an isoelectric point of 4.3 (McDonel, 1980b). The first 40 N-terminal amino acids of CPE 8-6 have been sequenced (Martin, 1991). There is homology to amino acids 3 through 42 of the amino acid sequence of the CPE-A (Czeczulin *et al.,* 1993). However, the first two amino acids (Met and Leu) of CPE-A are not present in the CPE 8-6. N-terminal sequencing of CPE-A has shown that approximately half of CPE-A molecules lack the first Met residue (Richardson and Granum, 1983). This residue may be lost during post-translational processing, suggesting CPE 8-6 may also lose the first two amino acids to post-translational processing. CPE 8-6 is only slightly more stable than CPE-A as inactivation occurs at 10 minutes instead of 5 minutes at 60°C.

CPE 8-6 has a LD_{50} (median lethal dose) in mice intraperitoneally (i.p.) of 39.0 μg/kg, an erythemal unit (EU) of 0.10 μg (Niilo, 1975), and erythemal activity of 9800 EU/μg protein (Lindsay *et al.*, 1985). These values are three times greater than those observed for CPE-A, which has a mouse LD_{50} (i.p.) of 112.0 μg/kg, an EU of 0.29 μg, and erythemal activity of 3410 EU/μg protein (Niilo, 1975). Interestingly, CPE-A has been shown to be activated threefold when treated with trypsin (Richardson and Granum, 1983) or chymotrypsin (Granum and Richardson, 1991) to yield a biological activity similar to that of CPE 8-6. However, trypsinization of CPE 8-6 results in no increase in activity (Lindsay *et al.*, 1985).

The CPE 8-6, like the CPE-A, is superantigenic. However, it is unknown if the superantigenic activity is linked to the enterotoxigenic activity in either of these two toxins. Both the protein and nucleic acid sequences for the CPE-A are known; however, the CPE 8-6 has not been similarly characterized.

The overall goal of this dissertation is to compare and contrast the type 8-6 enterotoxin from strain 8798 to that of the type A enterotoxin from strain 8239. The specific objectives were as follows:

1. To compare *C. perfringens* type A and type A 8-6 strains to determine enterotoxigenic activity using ELISA, Western immunoblot, Vero cell cytotoxicity, and PCR analysis.
2. To identify any differences in restriction enzyme profiles between type A and type A 8-6 strains using a series of DNA probes.
3. To determine the chromosomal location of the *cpeA* and *cpe86* genes.
4. To clone, sequence, and characterize the *cpe86* gene, and to compare and contrast it with the *cpeA* gene.

5. To compare and contrast the protein sequence and structure of the CPE-A and CPE 8-6 enterotoxins using protease digestion and matrix-assisted laser desorption (MALD) ionization mass spectrometry.

The results obtained will determine if there are indeed two different *C. perfringens* enterotoxins and how they differ. The characterization will provide information about the conserved and variable regions of the *C. perfringens* enterotoxin amino acid and nucleotide sequences. Since CPE 8-6 is more biologically active than CPE-A, sequence comparison may indicate the regions responsible for the alterations in activity. The results should provide a basis for future research on the mechanisms of pathogenicity and regulation of virulence expression in *C. perfringens.*

CHAPTER 2
REVIEW OF LITERATURE

Clostridium perfringens

Clostridium perfringens (*C. welchii*) is one of the most ubiquitous bacterial pathogens, occurring in soil, dust, water, marine sediments, all types of foods, and in the gastrointestinal tract of humans and animals. The pathogen is extensively studied due to its biomedical importance, having been implicated in myonecrosis (gas gangrene), foodborne illness, necrotic enteritis of infants, necrotic enteritis (pigbel or darmbrand), antibiotic associated diarrhea, acute sporadic diarrhea, sudden infant death syndrome, and a variety of veterinary diseases. The virulence of this organism is due to its remarkable ability to produce a variety of different toxins including two that cause foodborne illness, the type C β toxin and the type A enterotoxin (CPE-A). The type C β toxin causes a rare, often fatal necrotic enteritis that is associated with malnutrition and low trypsin levels in the gastrointestinal tract. The type A enterotoxin is the cause of a common human foodborne illness and is unique among enterotoxins in that it combines properties of two different toxin families: the enterotoxins and the pore forming toxins (McClane, 1994).

Bacteriology

Clostridium perfringens is a facultatively anaerobic, gram positive, non-motile, spore forming, rod-shaped bacterium that occurs singly or in pairs and is 0.6-2.4 x 1.3-1.9 μm in size. It can be distinguished from many other clostridia in being non-motile, able to reduce nitrate to nitrite, able to reduce sulfite to sulfide, and able to produce a stormy fermentation of lactose milk medium (Johnson, 1990). The organism grows well in media; however, its nutritional needs are complex, requiring 14 amino acids, 5 vitamins, uracil, and adenine as essential nutrients.

The organism is wide-spread due to several important properties. It has an unusually wide temperature of growth range, an extremely fast growth rate on many substrates, a relatively high tolerance to oxygen, and good survival through sporulation (Johnson, 1990). The temperature growth range is between 6 and 50°C, with the optimum growth range being between 43 and 45°C (Labbe, 1989). In nutrient rich media the doubling time can be as short as 8 minutes, making *C. perfringens* the fastest growing bacterium known (Johnson, 1990). Stringent anaerobic conditions are not necessary for growth and the optimum E_h is -200 mV.

In vitro sporulation of *C. perfringens* is difficult to achieve and is distinctly strain and medium dependent. The spores are large, oval, central or subterminal, and distend the cell. Spores lack appendages in contrast to many other clostridia. Approximately 75% of strains possess a capsule that is mainly composed of polysaccharides (Cato *et al.*, 1986). The temperature range for sporulation is narrower than that for growth, between 35 and 40°C. The spores can be divided into two classes, heat resistant and heat sensitive. The heat resistant spores have a D_{90} value of between 15 and 145 minutes and a Z value of 9 to 16°C. This class of spores usually requires a heat shock of 75-100°C for 5 to 20 minutes in order for germination to occur. The heat sensitive spores have a D_{90} value of between 3 and 5 minutes and a Z value of 6 to 8°C. Both classes can survive cooking of foods and may actually be stimulated for germination during heating. The differentiation in heat resistance in not well understood and the cause is unknown (Johnson, 1990).

Isolation and Enumeration of *C. perfringens*

Isolation of *C. perfringens* from a mixed culture involves enrichment in cooked meat medium with glucose for 4 to 6 hours at 45°C. This is then subcultured to iron milk medium at 37°C until clotting is observed. This culture is then streaked to egg-yolk agar or blood agar and incubated overnight at 37°C. *C. perfringens* colonies will have double zones of hemolysis and strong lecithinase activity. Selective media can also be used. Tryptose-sulfite-neomycin medium works well for the isolation from foods. This medium contains iron and sulfite, which upon reduction to sulfide causes the iron to form a black precipitate, resulting in black colonies. For confirmation of the species, purified colonies are inoculated into motility-nitrate agar, iron-milk medium, lactose-gelatin medium, and carbohydrate diagnostic broths.

Classification of *C. perfringens* Strains

C. perfringens strains may produce at least 13 and as many as 15 different toxins; however, any particular strain will produce only a defined subset of these toxins. Strains are classified into five types (A through E) based on the production and neutralization of 4 major toxin activities (alpha, beta, epsilon, and iota) with type specific antisera (Table 2-1). Since these toxin neutralization tests are laborious and require mice or guinea pigs, they are performed only by a few laboratories (Labbe, 1989).

Detailed physical and genetic maps have been made for the five types to determine if their classification by serology was a reflection of genomic organization (Canard *et al.*, 1992). The genetic maps did not reflect the

Table 2-1. Classification of *C. perfringens* by toxin type.

	Toxin Produced			
Type	α	β	ε	ι
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

From Johnson, 1990.

α = lethal, lecithinase; β = lethal, necrotic; ε = lethal, necrotic;
ι = lethal, necrotic.

serotype as there were as many differences in genomic map criteria between two type A strains as exist between strain types A and D, or between types A and E. Instead, the strains were found to be quite homogeneous, suggesting the phenotypic differences which are the basis of the serotyping system may stem from minor changes at the chromosomal level or the acquisition of new extra-chromosomal genetic information.

Type A strains may be further classified by serological typing based on differences in the capsular polysaccharides. Hobbs *et al.* (1953) developed the first serological typing classification for *C. perfringens* type A. Since then efforts by the United States, United Kingdom, and Japan have compiled well over 100 antisera for use in the International Serotyping Scheme for type A (Murrell, 1989). Serological typing can be used in the investigation of outbreaks to establish that strains isolated from different patients are the same, that strains isolated from the incriminated food and fecal samples are the same type, that a particular food is the source of the outbreak, or if outbreaks are related (Stringer, 1985). However, there are drawbacks to this classification scheme. The use of these antisera has resulted in an overall typability of only 82% (Murrell, 1989) and the lack of availability of the antisera limits the serotyping to a few large national reference laboratories (Labbe, 1989).

Plasmid profiling and bacteriocin production assays have been developed as less expensive and faster alternatives to the serotyping of type A strains (Eisgruber *et al.*, 1995; Mahony *et al.*, 1992). However, investigations of these assays have determined that approximately 25% of *C. perfringens* strains do not produce bacteriocins and 30% do not contain plasmids making these strains untypable by either the bacteriocin production or plasmid profiling assays.

C. perfringens Type A Foodborne Illness

Incidence of *C. perfringens* Foodborne Illness

Clostridium perfringens type A foodborne illness is one of the most common bacterial foodborne diseases in the USA. Between 1973 and 1987 there were 190 reported outbreaks and 12,234 cases of *C. perfringens* type A foodborne disease representing 11.2% of all bacterial foodborne disease cases in the USA (Bean and Griffin, 1990). Fatalities are rare and usually occur among the elderly or immunocompromised. However, the majority of cases of this foodborne disease are not officially reported due to the mild nature of the disease and difficulty in confirming cases. Official confirmation may be difficult for many reasons: the implicated food may not be available for testing, patients may not be available to test for *C. perfringens*, fecal specimens may be obtained too late, making isolates unrepresentative of the organisms present in the implicated food, or the *C. perfringens* is non-serotypable. Additionally, institutionalized geriatric individuals often have greater than 10^5 *C. perfringens* spores per gram of feces, and individuals in the same institution can carry the same serotype in the absence of illness (Jackson *et al.*, 1986). Overall, the incidence of *C. perfringens* type A foodborne illness would appear to be underreported, resulting in a gross underestimation of the true number of cases. The actual number of *C. perfringens* type A foodborne illness cases have been estimated at 652,000 per year in the USA (Todd, 1989).

Characteristics of *C. perfringens* Type A Foodborne Illness

Foodborne illness due to *C. perfringens* type A is caused by the enterotoxin designated CPE-A. Although Koch's postulates have not been used to formally prove CPE-A is the cause of foodborne illness, there are a large number of epidemiological studies that convincingly point to this conclusion. There was a strong positive correlation between the illness and the presence of CPE-A in patients feces at a level known to cause the disease in animals (Bartholomew *et al.*, 1985). Human volunteers fed purified CPE-A developed foodborne illness symptoms, and *C. perfringens* strains that produced CPE-A produced diarrhea in the volunteers while CPE-A negative strains did not. (Skelkvale and Uemura, 1977). Additionally, it was shown that the CPE-A effects on rabbit ileal loops can be neutralized with CPE-A specific antiserum (Hauschild *et al.*, 1971).

Illness usually occurs 8 to 24 hours after the consumption of food containing vegetative cells. The food vehicle is usually a meat product containing 10^5 to 10^6 CFU/g (Lund, 1990; Rood and Cole, 1991). The symptoms include diarrhea and severe abdominal pain. Nausea is a less common symptom, and fever and vomiting are unusual. Death can occur in elderly, immune-compromised and debilitated people. The symptoms are usually self-limiting and resolve in 12 to 24 hours. *C. perfringens* organisms are widely distributed and outbreaks do not usually occur in specific geographical locations. Susceptibility is equal for both sexes and is not age dependent beyond the infant stage. Most cases of the foodborne illness occur in the fall and winter for unknown reasons (Johnson, 1990).

The course of the foodborne illness begins when the contaminated food is ingested. The majority of the vegetative cells most likely die in the stomach due to exposure to acidity (McClane, 1994). However, some cells pass through the stomach to the small intestine where they sporulate; it is unknown what triggers the cells to sporulate. However, pathogens often modulate the expression of virulence factors in response to environmental stimuli

(Mekalanos, 1992). The harsh conditions of the stomach and small intestine are thought to initiate global changes in gene expression leading to the formation of a spore (McClane, 1994). The level of bile acids in the gut may play a role in the induction of these gene expression alterations (Heredia *et al.*, 1991).

CPE-A is produced during sporulation and does not contain a signal sequence. Therefore, it is not secreted from the sporulating cell immediately after synthesis, but is released upon the lysis of the mother cell into the intestinal lumen at the end of the sporulation cycle. This could explain why foodborne illness symptoms take 8-24 hours to develop after the ingestion of the vegetative cells. The cells must pass through the entire sporulation process, which takes 8 to 24 hours, before CPE-A is liberated into the intestines. The enterotoxin then binds to the intestinal epithelial cells and causes damage to the cells, resulting in fluid and electrolyte loss which is clinically manifested as diarrhea. An oral dose of 8 to 10 mg of toxin is required for illness to occur (Skelkvale and Uemura, 1977). This is a high amount of toxin when compared to other foodborne illness toxins such as the staphylococcal A enterotoxin which only requires 1 μg to cause illness (Johnson, 1990). This disease is thought to be self-limiting and mild due to the flushing of CPE-A and *C. perfringens* cells out of the intestine by the diarrhea. In addition, CPE-A affects the older intestinal cells, the villus tip cells, which can be quickly replaced during the normal turnover of intestinal cells.

Food Vehicles

Meat and poultry products were the most common food vehicle for *C. perfringens* type A foodborne illness outbreaks between 1973 and 1987 in the United States (Bean and Griffin, 1990). Beef accounted for over 26%, chicken and turkey for almost 15%, and Mexican foods for 12% of these outbreaks. Improper food holding temperatures contributed to the cause of 97% of the outbreaks where causative factors were reported. While inadequate cooking contributed to 65%, poor personal hygiene and contaminated equipment each contributed to about 27% of the outbreaks. In some cases several factors were reported as the cause of an outbreak.

Prevention and Control of *C. perfringens* Type A Foodborne Illness

Vegetative cells of *C. perfringens* type A are relatively heat tolerant, having an optimal growth temperature between 43 and 45°C and surviving to 60°C. Interestingly, as the growth temperature of the cells increases so does their heat resistance. Under optimal conditions the cells can double in as little as 8 minutes. The spores are usually very heat resistant and can be "heat activated" by heating at a sublethal temperature resulting in increased germination rates of the spores (Labbe, 1989).

These properties of vegetative cells and spores of *C. perfringens* make them well adapted to taking advantage of sub-optimal food preparation and storage conditions. When a food is not completely cooked, surviving spores can germinate and multiply. If the food is improperly cooled and held, the cells can multiply rapidly. Alternatively, if a food is contaminated by vegetative cells or spores after cooking due to poor hygiene or contaminated equipment, the cells can multiply rapidly in improper storage and holding temperatures.

C. perfringens type A foodborne illness can be controlled and prevented by thoroughly cooking foods, especially large cuts or pieces of meat, such as roast pork or large portions of corned beef (Centers for Disease Control, 1994). These are frequently the cause of illness, since high internal temperatures sufficient to kill *C. perfringens* spores are not always reached. Between 1976 and 1980 in the USA, foods prepared in restaurants (56%), schools (11%), camps (5%), nursing homes (3%), and prisons (3%) were implicated in the majority of outbreaks. Foods prepared in private homes were only implicated 3% of the time. This points to large batches of food prepared in institutional settings not being properly cooked, cooled, and held as the source of illness (Shandera *et al.*, 1983). Once cooked, foods need to be quickly cooled (within 2-3 hours) and stored or held at nonpermissive temperatures (below 10°C or above 70°C) to prevent vegetative growth of *C. perfringens*. Also, cooked, cooled foods should be reheated to a minimum internal temperature of 75°C immediately before serving (Lund, 1990).

Reservoirs of *C. perfringens* Type A

C. perfringens type A strains are part of the microflora of soil and are found at the level of 10^3 to 10^4 CFU/g of soil in virtually all soil samples tested (Labbe, 1989; Smith and Gardner, 1949; Taylor and Gordon, 1940). The organism is found in about 50% of all raw or frozen meat and poultry products, and has been found in many types of processed foods. Feces from healthy humans can contain 10^3 to 10^4 spores of *C. perfringens* type A per gram and the organism has been found in the intestine of virtually every animal tested. The widespread distribution of this organism is thought to explain the many incidences of *C. perfringens* type A foodborne illness.

Unfortunately, few studies have examined the distribution of *C. perfringens* type A strains that carry the *cpeA* gene which must be present for foodborne illness to occur. It is unknown where the *cpeA* carrying strains reside in the environment amongst the many strains of non-enterotoxigenic *C. perfringens*. Early studies attempted to differentiate the enterotoxigenic and non-enterotoxigenic strains by identifying the heat resistant strains (Smith and

Gardner, 1949). It was originally thought that heat resistant strains (surviving at 100°C for 60 minutes) were the only strains that caused foodborne illness in England (Hobbs *et al.*, 1953). These heat resistant strains were unlike the classical heat sensitive (surviving at 100°C for 10 minutes) strains. However, in the United States many outbreak strains were like the classical heat sensitive strains (Hauschild and Thatcher, 1967; Hathaway, 1990), and it was later shown that outbreaks in England were also caused by heat sensitive strains (Sutton and Hobbs, 1965). Therefore, heat resistance is not a reliable indicator of enterotoxigenicity.

Van Damme-Jongston *et al.* (1989) demonstrated that less than 6% of strains isolated from different species of animals carry the *cpeA* gene, while Kokai-Kun *et al.* (1994) found 3.5% of isolates from animals carry the gene. Van Damme-Jongsten *et al.* (1990) determined 59% of the isolates from confirmed outbreaks of food poisoning carried the gene. This result was unexpected, and the authors suggested that contaminating *cpeA* negative strains of *C. perfringens* were isolated instead of the causative organism. Saito (1990) found enterotoxigenic strains in 6% of healthy food handlers, 12% of oysters, 10% of water samples, and in none of the chicken, swine, or cattle samples. The environmental reservoirs of enterotoxigenic *C. perfringens* type A strains remain unclear.

Recently, veterinary strains of *C. perfringens* have been found to carry the *cpeA* gene on a plasmid which makes the issue of reservoirs more complex (Cornillot *et al.*, 1995). The chromosomal *cpeA* gene was only found in human foodborne illness strains, while the plasmid-borne *cpeA* gene was only found in strains of animal origin. It is unclear if strains that carry the plasmid-borne *cpeA* gene can cause foodborne illness. Studies using newer technologies such as the polymerase chain reaction amplification specific for the *cpeA* gene and DNA gene probes could help to provide insight into the environmental reservoirs. Once the reservoirs are identified, the determination of how food becomes contaminated with *C. perfringens* will be easier, and better control and prevention systems could be identified and utilized.

Identification of *C. perfringens* Type A Foodborne Illness Outbreaks

Public health officials use several criteria to establish an outbreak of *C. perfringens* type A foodborne illness. The incubation time, type of symptoms, and type of food involved are analyzed along with laboratory results. The laboratory results should identify greater than 10^5 *C. perfringens* cells per gram of implicated food, greater than 10^6 *C. perfringens* spores per gram of feces from an affected individual, the same serotype of *C. perfringens* in all ill individuals, or the same serotype in the implicated food and in the fecal isolates from ill individuals (McClane, 1992).

Unfortunately, these criteria have drawbacks as identified in the Incidence of *C. perfringens* Foodborne Illness section. In light of these limitations, it has been suggested that additional diagnostic criteria be taken into account, such as demonstrating the enterotoxin in feces of ill individuals or demonstrating *cpeA* positive *C. perfringens* strains in the food and feces of a suspected outbreak (McClane, 1992).

Assays for the Identification of *C. perfringens* Enterotoxin

Several serological assays have been developed for the detection of CPE-A in feces and are good indicators of *C. perfringens* foodborne illness. Both enzyme linked immunosorbent and reversed passive latex agglutination assays are commercially available. However, there are some limitations to these assays. First, fecal samples must be collected within 48 hours of the onset of illness to provide accurate results (Bartholomew *et al.*, 1985). Second, these assays require that the isolated *C. perfringens* strains sporulate *in vitro*, which can be difficult to achieve.

Alternative assays identify the presence of the *cpeA* gene in *C. perfringens* isolates, eliminating the need for *in vitro* sporulation. Probes homologous to portions of the *cpeA* gene can be used to successfully identify strains carrying the gene (Van Damme-Jongsten *et al.*, 1990). Additionally, the polymerase chain reaction can identify *cpeA* gene carrying strains (Baez and Juneja, 1995; Saito *et al.*, 1992) in food and feces without isolation of the organism. However, these gene detection assays only identify potentially enterotoxigenic strains since the presence of the CPE-A protein itself is not established.

Medical Importance of the *C. perfringens* Type A Enterotoxin Other than Foodborne Illness

In addition to foodborne illness, *C. perfringens* type A enterotoxin has been implicated in antibiotic-associated diarrhea, infectious diarrhea, SIDS, and in gastrointestinal diseases of many types of animals. CPE-A has been implicated in antibiotic-associated diarrhea where patients suffer from prolonged, profuse, and sometimes bloody diarrhea after antibiotic treatment (Borriello *et al.*, 1984). It is not known whether the antibiotics permit infection by *C. perfringens* or allow the overgrowth of small numbers of the organism that may be part of the normal microflora of the gut. Additional cases of this prolonged and sometimes bloody diarrhea have occurred without the prior use of antibiotics (Borriello *et al.*, 1985; Larson and Borriello, 1988). These cases may be infectious diarrhea due to *C. perfringens* type A. Both the antibiotic-associated diarrhea and the infectious diarrhea differ from foodborne illness in that the duration of symptoms is approximately 11 days. This is unlike the duration of foodborne illness (24 hours) and much more like the duration of infectious diarrhea caused by *Salmonella* or *Campylobacter*. Additionally, the antibiotic-associated and infectious diarrhea predominately affect the elderly, can produce blood and mucus in the stool, and can frequently produce abdominal pain whereas type A foodborne illness does not.

C. perfringens type A enterotoxin has been implicated as a possible trigger for SIDS (Lindsay *et al.*, 1993). *C. perfringens* type A has been found in over 80% of fecal samples from SIDS cases and in less than 2% fecal samples from control cases. There is a strong positive correlation of the presence of enterotoxin with the presence of the organism. Ileal tissue from the SIDS cases resembles the ileal tissue of animals treated with the enterotoxin. Additionally, the enterotoxin is a superantigen (see later) and, as a result of the large T lymphocyte stimulation, large amounts of cytokines are produced which can cause shock and respiratory interference. Lindsay *et al.* (1994) hypothesized that the enterotoxin can trigger SIDS in a predisposed infant. The predisposition may be due to fetal insult *in utero* or developmental abnormalities. An initial respiratory or gastrointestinal infection is usually involved, which may serve to increase the infant's vulnerability. A secondary bacterial infection involving *C. perfringens* (or another superantigen producing organism) then causes the release of superantigenic enterotoxin. The superantigen causes massive cytokine release in the vulnerable infant, which induces shock and respiratory interference leading to hypoxia, brain astrogliosis, and pulmonary edma. The final result is death of the infant.

C. perfringens Type A Enterotoxin Composition and Structure

The type A enterotoxin (CPE-A) is a 319 amino acid protein that has a molecular mass (M_r) of 35,317 (Czeczulin *et al.* 1993), which is processed by trypsin to a more active form of M_r 31,472. The removal of the amino terminal 25 amino acids results in a threefold increase of biological activity (Rood and Cole, 1991). The amino acid sequence of CPE-A has no significant homology to any other protein in the Genbank/EMBL database (Czeczulin *et al.*, 1993) suggesting the amino acid sequence is unique. CPE-A is very hydrophobic, containing of 43% hydrophobic amino acids, resulting in a low water solubility of 4 mg/ml. CPE-A protein contains only one cysteine residue and therefore does not form stabilizing disulfide bridges. The lack of a disulfide bridge contributes to the heat instability of the protein, which is inactivated by heating at 60° C for 5 minutes. Little is known of the CPE-A three dimensional structure except that it is composed of approximately 80% β-sheet and 20% α-helix structure as determined by circular dichroism (Granum and Harbitz, 1984). X-ray analysis of the protein could provide more information about the three dimensional structure of the enterotoxin; however, suitable crystals of the protein have not yet been produced, even though the protein is prone to aggregation and can cause inclusion bodies to form in the cell (McClane, 1994). CPE-A aggregates (forms quaternary structure) *in vivo* and *in vitro*. The protein also binds sodium dodecyl sulfate (SDS) anomalously (0.39 g/g of protein) resulting in aggregation of the protein in SDS polyacrylamide gel electrophoresis (PAGE).

Structure and function studies of the type A enterotoxin molecule have determined that the polypeptide is most likely composed of two separate functional regions (Figure 2-1). These regions are responsible for receptor binding and cytotoxicity, with the binding region being the best characterized. Receptor binding activity has been localized to the 30 carboxy terminal amino acids (Hanna *et al.*, 1991). Synthetic and recombinant fragments representing amino acids 290-319 of CPE-A exhibit receptor binding activity, but no cytotoxic activity. These fragments do not insert into the membranes. Therefore, receptor binding in itself does not result in cytotoxicity and later steps are required for the cytotoxicity to occur. An even larger recombinant CPE-A fragment consisting of amino acids 171-319 retains receptor binding activity, but is still biologically inactive (Hanna *et al.*, 1989). Therefore, amino acids 26-171 must contain the cytotoxic activity, since trypsin removal of amino acids 1-25 actually increases cytotoxicity threefold (Granum *et al.*, 1981; Hanna *et al.*, 1992). The increase of cytotoxicity after trypsinization is not due to improved binding function of the CPE-A, but is an actual increase in cytotoxic activity per bound CPE-A molecule (Hanna *et al.*, 1992). This increase in cytotoxic activity after trypsinization may be due to a conformational change in the nearby cytotoxic functional region after amino acids 1-25 are removed (Hanna *et al.*, 1992). Amino acids 1 through 25 do not seem to have a function related to CPE-A action and do not comprise a signal sequence (Czeczulin *et al.*, 1993).

The *C. perfringens* Type A Enterotoxin is Superantigenic

It has recently been determined that CPE-A is a superantigen. Superantigens have been found to stimulate as many as 1 in 5 T lymphocytes in contrast to a classical antigen that stimulates 1 in 10,000 T lymphocytes. The T cell stimulation causes the induction of a cytokine cascade (interferon-γ, tumor necrosis factor-β, and interleukins) which in turn can cause decreased blood pressure, shock, respiratory interference, multiple organ failure, and death (Kotzin *et al.*, 1993). Superantigens can activate many T cells but the

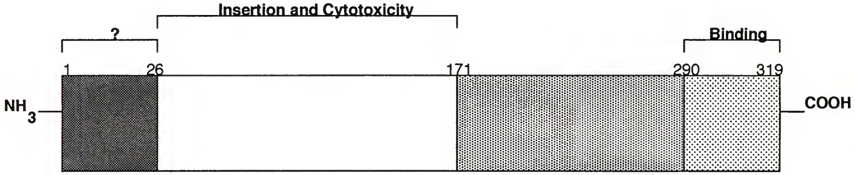

Figure 2-1 Map of the functional regions of the *C. perfringens* type A enterotoxin. Adapted from McClane 1994.

majority of the T cells are useless in fighting an infection in humans. Additionally, some of these activated cells may actually cause an autoimmune attack. Superantigens can also trigger the death of cells they activate, further damaging the immune system. CPE-A stimulates human T cell receptors Vβ6.9 and Vβ22 and weakly stimulates Vβ24, Vβ21, Vβ18, Vβ5, and Vβ6.1-5 (Bowness *et al.*, 1992). CPE-A can also out-compete the staphylococcal enterotoxin A for binding to class II major histocompatability complex molecules (Lindsay and Johnson, unpublished observations).

It is unknown if the superantigenicity is linked to the enterotoxigenicty of CPE-A. The relationship between the emetic response and superantigenicity in the well characterized superantigenic staphylococcal enterotoxins (SEs) is currently being analyzed. It is unclear if the emesis caused by the SEs is a direct consequence of a particular aspect of the superantigenic activity or is unrelated (Betley and Harris, 1994).

Coincidentally, CPE-A has much in common with the SEs. CPE-A is similar in size to the SEs, is superantigenic (Bowness *et al.*, 1992), and has been shown to be carried both on the chromosome and on a plasmid (Cornillot *et al.*, 1995) as the SEs have been. The functional half life of the *cpeA* mRNA in sporulating cells has been reported to be as long as 58 minutes (Labbe and Duncan, 1977), and the mRNA of the SEs have also been found to have an exceptionally long half-life (Katsuno and Kondo, 1973). Additionally, CPE-A is produced in varying amounts by different strains as has been observed with the SEs.

Mode of Action of the *C. perfringens* Type A Enterotoxin

CPE-A has a unique method of action that includes binding to mammalian plasma membranes, insertion or entrapment in the membrane, formation of a large complex in the membrane, alterations in the plasma membrane permeability for small molecules, and subsequent DNA, RNA, and protein synthesis inhibition, large molecule permeability, and morphological damage or cells death.

Binding of CPE-A to a Cellular Receptor

The first step in CPE-A action is the rapid binding of the molecule to susceptible cells in a specific and saturable manner (McClane *et al.*, 1988; McDonel, 1980a). Saturation of the receptors occurs in 5 to 10 minutes. The proteinaceous receptor occurs on a wide range of cell types from many species of animals, and is present in great numbers (10^6 per cell) on the surface of these cells (Wnek and McClane, 1986), suggesting the receptor is important for a normal physiologic role in mammalian cells. However, this molecule is not essential for the viability of all mammalian cells since it is not present on all cells. The identity of the receptor is unknown; however, CPE-A has been shown to bind and complex with a 45 to 50 kDa plasma membrane protein that is thought to be the receptor (Wieckowski *et al.*, 1994).

Permanent Membrane Association

After binding to the plasma membrane, the CPE-A is not internalized into the cell, but remains associated with the membrane. It cannot be dissociated from the membrane using proteases, chaotropic salts or EDTA (Hanna and McClane, 1991; McDonel, 1980a). The association kinetics of CPE-A with membranes suggests a rapid two step process which most likely represents CPE-A binding followed quickly by a second process such as insertion (McClane *et al.*, 1988). Once the CPE-A is associated with membranes it acquires the amphiphilic characteristics of an inserted toxin (Wieckowski *et al.*, 1994). These results suggest an insertion of the CPE-A into the membrane or a conformational change of the receptor and CPE-A molecule complex (McClane, 1994).

Large Complex Formation

Next, the membrane associated CPE-A complex most likely becomes associated with a 70 kDa membrane protein to form a 160 kDa large complex (Wnek and McClane, 1989). This large complex formation does not occur at 4°C and because membrane fluidity is reduced at 4°C, membrane diffusion may be necessary for the large complex to form (McClane and Wnek, 1990). Since both the receptor binding and insertion can occur at 4°C, the large complex is most likely formed after these events. Additionally, CPE-A cytotoxicity is blocked at 4°C, suggesting that the large complex formation is necessary for toxicity.

Membrane Permeability Alterations

After CPE-A receptor binding, insertion, and large complex formation, alterations in membrane permeability occur. These alterations are observed within 5 to 15 minutes of the addition of CPE-A (McClane, 1984; McClane and McDonel, 1980). Initially, the membranes are only permeable to molecules of less than 200 daltons, which includes ions and amino acids. The permeability is non-selective, and allows the molecules to freely pass into or out of the cell. Since the permeability alterations occur so rapidly, it is thought that CPE-A directly affects the membrane without the use of intermediate messengers or activation of metabolic cascades (McClane, 1994). As membrane permeability develops, intracellular ion and other small molecule concentrations are altered, most likely causing the inhibition of incorporation of precursors into DNA, RNA and protein that occurs within 15 minutes of CPE-A treatment (Hulkower *et al.*, 1989). With time, permeability alterations develop for molecules up to 5 kDa, which does not change until cell lysis occurs. An influx of Ca^{+2} is required for membrane permeability and cell morphology alterations. Elevated Ca^{+2} levels most likely lead to the collapse of the cytoskeleton, which in turn could contribute to the observed morphological changes of the villus tip enterocytes.

CPE-A Action is Unique

CPE-A has a unique mechanism of action that combines properties of two different toxin families: the enterotoxins and the pore-forming toxins (McClane, 1994). The enterotoxins include cholera toxin, *C. difficile* toxin A, and Shiga toxin, while the pore-forming toxins include staphylococcal α toxin, and streptolysin O. CPE-A is similar to other enterotoxins in that it produces fluid and electrolyte loss from the gastrointestinal tract. CPE-A is distinguished from other enterotoxins by the production of rapid intestinal tissue damage by directly altering the permeability of the brush border membrane of intestinal epithelial cells. Other enterotoxins either do not produce visible damage or require much longer periods of toxin treatment to produce visible damage and do not directly alter cell permeability. CPE-A also differs from other enterotoxins since it does not affect intestinal cAMP levels, as other enterotoxins do. CPE-A inhibits intestinal glucose absorption while other enterotoxins do not. CPE-A is not heat stable in contrast to some other enterotoxins, such as the *Escherichia coli* heat stable and staphylococcal enterotoxins.

CPE-A is similar to the pore forming toxins in that they both cause rapid and direct damage to plasma membrane permeability. However, CPE-A differs from the pore forming toxins in requiring intimate involvement of mammalian proteins (i.e., a protein receptor) for its action. The action of pore forming toxins is thought to be membrane protein independent (McClane, 1994). It is unclear if CPE-A actually forms a pore in the plasma membrane or if it inserts into membranes and affects membrane permeability through another mechanism.

Molecular Biology of the *C. perfringens* Type A Enterotoxins

Cloning and Sequencing of the *C. perfringens cpeA* Gene

The cloning of the entire *cpeA* gene has proved to be a time consuming and difficult task, prompting early speculation that the cloning of the *cpeA* gene in *E. coli* was highly unfavorable (Granum and Stewart, 1993). Several independent groups have attempted to clone the *cpeA* gene using different approaches. Van Damme-Jongsten *et al.* (1989) used a synthetic oligonucleotide probe based on the protein sequence (Richardson and Granum, 1985) to identify the gene. The probe was based on amino acids 125-134 since there was minimal degeneracy of the genetic code in this region. To further reduce the 2084-fold degeneracy of this probe, the high A+T content of *C. perfringens* DNA was taken into account. By choosing an adenine or thymine for the third base of codons the degeneracy of the probe was reduced to fourfold. *C. perfringens* genomic DNA was then digested to completion with several restriction enzymes, subjected to agarose gel electrophoresis, and Southern blotted using the probe for detection. The *Rsa*I and *Hin*dIII restriction enzymes produced probe-positive fragments of approximately 1 Kb. Therefore, these digestions were separated by electrophoresis and fragments corresponding to 0.8 to 1.2 Kb were isolated and ligated into the pUC19 vector. Two clones containing the entire *cpeA* sequence were obtained. However, the whole gene was not cloned, and minimal flanking DNA sequence was obtained.

Iwanejko *et al.* (1989) also used a synthetic oligonucleotide probe based on the protein sequence. This group chose amino acids 9-17 for the probe, resulting in 512-fold degeneracy. To reduce the degeneracy to fourfold, the convention that G-T interactions are neutral in terms of base pairing was applied. *C. perfringens* genomic DNA was partially digested with *Hin*dIII and DNA fragments corresponding to 6-10 Kb were isolated and ligated into the *E. coli* vector pHG165 (Stewart *et al.*, 1986). A probe-positive 6.8 Kb clone was obtained that contained significant sequence 5' to the *cpeA* gene and most, but not all, of the *cpeA* gene.

Hanna *et al.* (1989) used an anti-CPE-A monoclonal antibody to screen for clones of the *cpeA* gene. Genomic *C. perfringens* DNA was sheared randomly and *Eco*RI linkers were added to the fragments which were then cloned into λgt11 bacteriophage vector. One plaque was obtained that reacted with the monoclonal antibody. This clone contained only the 3' half of the *cpeA* gene (amino acids 171-320) and 1.8 Kb 3' to the gene.

Iwanejko and Stewart (Granum and Stewart, 1993) attempted to clone the entire *cpeA* gene by using a complete *Eco*RI digestion of genomic DNA. *Eco*RI does not cut within the *cpeA* gene and resulted in a 10 Kb probe-positive fragment in Southern analysis. This attempt to clone the entire gene was also unsuccessful.

It is unclear why these varied cloning strategies by independent laboratories were unsuccessful. However, in 1993, Czeczulin *et al.* (1993) successfully cloned the entire *cpeA* gene using a rather straightforward methodology. Genomic DNA, digested with *Xba*I, produced a positive band of 5.7 Kb when Southern blotted and detected with a 0.5 Kb *cpeA* DNA fragment probe. Therefore, *C. perfringens* genomic DNA was digested with *Xba*I, subjected to agarose gel electrophoresis, and DNA bands corresponding to 4 to

7 Kb were cut out of the gel and purified. This DNA was then cloned into the *E. coli* vector, pUC19. Recombinant plasmids were then transformed into *E. coli* DH5α. The transformants were screened with the 0.5 Kb probe. A probe positive clone was obtained that carried a 5.7 Kb *C. perfringens* DNA insert. This insert was analyzed by nucleotide sequencing and was found to contain the entire coding region for the *cpeA* gene.

The nucleic acid sequence of the *cpeA* gene codes for a 319 amino acid protein of 35,317 Da (Figure 2-2). Microheterogenecity was observed in the sequence when compared to the earlier sequencing of partial clones and more extensive differences were observed in the downstream region of the gene. A possible rho independent stem loop transcription terminator consisting of a stem-loop structure followed by an oligo-T tract was identified for the first time downstream of the 3' end of the gene.

Expression of Recombinant *C. perfringens* Enterotoxin

Expression of recombinant CPE-A protein was achieved in *E. coli* by Czeczulin *et al.* (1993). Western immunoblot analysis revealed that cell lysates of the recombinant *E. coli* contained a 35 kDa protein that comigrated with the CPE-A protein and reacted with the polyclonal antibodies specific for the purified CPE-A. The rCPE-A was localized in the periplasm of the *E. coli.* This result was unexpected for two reasons. First, CPE-A accumulates intracellularly in the *C. perfringens* cell and is not released until the lysis of the mother cell. Second, no typical signal sequence was identified (Czeczulin *et al.*, 1993). The 5' codons of the *cpeA* sequence directly code for the mature N-terminal amino acids of the protein, and there are in-frame termination codons directly upstream of the *cpeA* initiation codon. The transport mechanism by which the CPE-A is moved to the *E. coli* periplasm is unknown.

```
1       ATGCTTAGTA ACAATTTAAA TCCAATGGTG TTCGAAAATG CTAAAGAAGT
        M  L  S  N   N  L  N   P  M  V   F  E  N  A   K  E  V
51      ATTTCTTATT TCTGAGGATT TAAAAACACC AATTAATATT ACAAACTCTA
         F  L  I   S  E  D  L   K  T  P   I  N  I   T  N  S  N
101     ACTCAAATTT AAGTGATGGA TTATATGTAA TAGATAAAGG AGATGGTTGG
          S  N  L   S  D  G   L  Y  V  I   D  K  G   D  G  W
151     ATATTAGGGG AACCCTCAGT AGTTTCAAGT CAAATTCTTA ATCCTAATGA
        I  L  G  E   P  S  V   V  S  S   Q  I  L  N   P  N  E
201     AACAGGTACC TTTAGCCAAT CATTAACTAA ATCTAAAGAA GTATCTATAA
         T  G  T   F  S  Q  S   L  T  K   S  K  E   V  S  I  N
251     ATGTAAATTT TTCAGTTGGA TTTACTTCTG AATTTATACA AGCATCTGTA
          V  N  F   S  V  G   F  T  S  E   F  I  Q   A  S  V
301     GAATATGGAT TTGGAATAAC TATAGGAGAA CAAAATACAA TAGAAAGATC
        E  Y  G  F   G  I  T   I  G  E   Q  N  T  I   E  R  S
351     TGTATCTACA ACTGCTGGTC CAAATGAATA TGTATATTAT AAGGTTTATG
         V  S  T   T  A  G  P   N  E  Y   V  Y  Y   K  V  Y  A
401     CAACTTATAG AAAGTATCAA GCTATTAGAA TTTCTCATGG TAATATCTCT
          T  Y  R   K  Y  Q   A  I  R  I   S  H  G   N  I  S
451     GATGATGGAT CAATTTATAA ATTAACAGGA ATATGGCTTA GTAAAACATC
        D  D  G  S   I  Y  K   L  T  G   I  W  L  S   K  T  S
501     TGCAGATAGC TTAGGAAATA TTGATCAAGG TTCATTAATT GAAACTGGTG
         A  D  S   L  G  N  I   D  Q  G   S  L  I   E  T  G  E
551     AAAGATGTGT TTTAACAGTT CCATCTACAG ATATAGAAAA AGAAATCCTT
          R  C  V   L  T  V   P  S  T  D   I  E  K   E  I  L
601     GATTTAGCTG CTGCTACAGA AAGATTAAAT TTAACTGATG CATTAAACTC
        D  L  A  A   A  T  E   R  L  N   L  T  D  A   L  N  S
651     AAATCCAGCT GGTAATTTAT ATGATTGGCG TTCTTCTAAC TCATACCCTT
         N  P  A   G  N  L  Y   D  W  R   S  S  N   S  Y  P  W
701     GGACTCAAAA GCTTAATTTA CACTTAACAA TTACAGCTAC TGGACAAAAA
          T  Q  K   L  N  L   H  L  T  I   T  A  T   G  Q  K
751     TATAGAATCT TAGCTAGCAA AATTGTTGAT TTTAATATTT ATTCAAATAA
        Y  R  I  L   A  S  K   I  V  D   F  N  I  Y   S  N  N
801     TTTTAATAAT CTAGTGAAAT TAGAACAGTC CTTAGGTGAT GGAGTAAAAG
         F  N  N   L  V  K  L   E  Q  S   L  G  D   G  V  K  D
851     ATCATTATGT TGATATAAGC TTAGATGCTG GACAATATGT TCTTGTAATG
          H  Y  V   D  I  S   L  D  A  G   Q  Y  V   L  V  M
901     AAAGCTAATT CATCATATAG TGGAAATTAC CCTTATTCAA TATTATTTCA
        K  A  N  S   S  Y  S   G  N  Y   P  Y  S  I   L  F  Q
951     AAAATTTTAATATTTTAAAATAATATAATCAAATTAATTTACAAAAGACAGTAT
         K  F  *                                     SL
        GTAATATTAAATTATTACATACTGTCTAATTTTTTT
                         SL
```

Figure 2-2. Nucleotide and amino acid sequence of *C. perfringens* type A enterotoxin (Czeczulin *et al.*, 1993). A 3' stem loop (SL) structure is underlined and may be involved in transcriptional termination or message stability.

Sporulation and Expression of *C. perfringens* Type A Enterotoxin

The production of CPE-A has been linked to the sporulation of the *C. perfringens* cell. Sporulation of the bacterial cell is divided into 8 stages beginning with stage 0, the vegetative cell, and ending with stage VII, the liberation of the mature spore. Duncan *et al.* (1972) isolated several sporulation mutants of a *cpeA* positive strain. Mutants blocked from sporulation at stage 0 did not continue to produce CPE-A, while mutants blocked at later stages of sporulation (stages III, IV and V) continued to produce CPE-A. Since late stage sporulation mutants produce the CPE-A, it seems likely that the gene requires an unknown stage I or II sporulation gene for activation (Rood and Cole, 1991). This study demonstrated a direct link between sporulation and the production of CPE-A.

However, more recent studies have shown that CPE-A production is not strictly dependent upon sporulation. Sporulating *C. perfringens* cells produce approximately 65 μg of CPE-A per ml of cells. CPE-A can account for about 13% of the total cell lysate protein under sporulating conditions (Czeczulin *et al.*, 1993). Vegetative *C. perfringens* cells can also produce CPE-A; however, they are estimated to produce less than 50 ng of CPE-A per ml of cells. This represents less than 0.05% of the total cell lysate protein. The vegetative production of trace amounts of CPE-A may occur due to low constitutive levels of production. Recombinant CPE-A produced by overnight cultures of *E. coli* represented approximately 0.2% of total cell lysate protein or 2.7 μg of CPE-A per ml of cells (Czeczulin *et al.*, 1993). Recombinant CPE-A is produced in *E. coli* under non-sporulating conditions (since this organism does not sporulate) from a clostridial promoter. These results demonstrate that while sporulation is not necessary for CPE-A to be expressed, it does greatly promote the expression of the protein in *C. perfringens*.

The molecular regulation of the *cpeA* gene during vegetative growth and sporulation is not understood in detail. The expression of CPE-A protein is associated temporally and genetically to sporulation. However, less than 6% of *C. perfringens* carry the *cpeA* gene (McClane, 1994; Van Damme-Jongsten *et al.*, 1989). These strains can produce varying amounts of CPE-A and their ability to do so can vary (Brynestad *et al.*, 1994).

To examine the regulation of the *cpeA* gene, Melville *et al.* (1994) cloned the *cpeA* promoter region from several strains of *C. perfringens*. These promoters were then fused to the *E. coli gusA* gene (coding for β-glucuronidase) in order to measure *cpeA* gene expression in *C. perfringens* and *Bacillus subtilis*. Expression of the reporter gene activity indicated that in *C. perfringens* the synthesis of *cpeA* mRNA and CPE-A was suppressed during vegetative growth and greatly increased 1 hour after the end of exponential phase growth. Therefore, the induction of *cpeA* expression occurs in early stationary phase. The accumulation of both *cpeA* mRNA and CPE-A at this time indicates that CPE-A synthesis is limited by the rate of transcription of the *cpeA* gene. It is unknown if the accumulation of mRNA is due to an increased rate of synthesis or to stabilization of the mRNA. The functional half life of the *cpeA* mRNA in sporulating cells has been reported to be as long as 58 minutes (Labbe and Duncan, 1977).

Two types of CPE-A promoters were identified in foodborne isolates of *C. perfringens* (Figure 2-3). The first promoter type has a 45 base pair insertion that is not present in the second promoter type. The starting point of transcription occurred at 197 bp upstream of the translational start site regardless of the promoter type (with or without the 45 bp insertion). This promoter region shows no similarity to any known bacterial promoter consensus sequences, including those recognized by the sporulation specific sigma factors of *B. subtilis*.

When CPE-A is expressed in *B. subtilis*, there is recognition of a promoter site within the 45 bp insertion (50 bp upstream of the start site used in *C. perfringens*). This insertion sequence has a potential vegetative type promoter. If the promoter is recognized by the major vegetative RNA polymerase of *B. subtilis*, any sporulation dependent regulation could be obscured. However, both promoter types were used in vegetative *B. subtilis* cells and both showed threefold induction of *cpeA* expression in early stationary phase. Therefore, the threefold induction must occur at a promoter other than the one within the 45 bp insertion since the promoter type without the insertion also was induced at early stationary phase.

Expression of the *cpeA* gene was also tested in *B. subtilis* strains that were mutated in the Spo0A, AbrB, σ^B, σ^E, and σ^H proteins to determine if any were important in the expression of *cpeA*. These proteins control stationary phase gene expression in *B. subtilis*. However, none of these *B. subtilis* mutants had an effect on the expression of the *cpeA* promoter, suggesting that *C. perfringens* does not regulate the expression of *cpeA* with proteins that are similar to the *B. subtilis* proteins Spo0A, AbrB, σ^B, σ^E and, σ^H.

There is some evidence that the *cpeA* gene is regulated by some unidentified sigma factor. *C. perfringens* types A, C, and D can produce the enterotoxin (Hatheway, 1990). However, the enterotoxins from types C and D have not been completely characterized and have not been implicated in any cases of foodborne illness. It is unclear as to why only type A strains have caused human foodborne illness. Type B has never been shown to produce an enterotoxin. Collie *et al.* (1995) transformed plasmids carrying the *cpeA* gene

```
1       GCTTAACTAT TCTTGATAGT TATCTGGTAA AATATTAATT TTAAATCCTT

51      GTAAAACCAA GGAAATTAAG GTTGCTATAG AAAGCCATAA CCCATAATTC

101     TTAAAACGTG ATTTATTTAT TATAATATAT CACTCTCCTT GTTTATAATA

151     TATAATATTA TGTTTAGTGA AATTATGTTA ATATACATTG TCTATTTTTA
                                                  Insertion
201     TAGTTTAACT ATAAAATAAG TAGTATAGTC TTTATTTCTT CTTTTATATT

251     AATTAACATT TCAACTTGAT CTCTTTAACG TATATCTCTT TTATTACCCA
                                             *
301     AGCTTTAATT CCTTCAGCAT TAATATCATA AAATGTCCAT GTAGAAATAT

351     ATTCAAGATT ATTAAAGATA TATATTTTAT TTAATATTTT GTTAATACTT

401     TAAGGATATG TATCCAAAAT AAAAACTTTT AAATAATATA TTATATAAAA

451     AAAATTAGAA ATAAGGAGAT GTTAATTATA ATATG
                        SD                 MET
```

Figure 2-3. *C. perfringens* type A enterotoxin promoter region (Czeczulin *et al.*, 1993; Melville *et al.*, 1994). The initial methionine codon for the *cpeA* structural gene is marked MET. The proposed Shine-Dalgarno (SD) translational recognition sequence is identified. The star represents the 5' end of the mRNA. The 45 base pair insertion sequence that is not present in all strains is noted.

into *cpeA* negative *C. perfringens* types A, B, and C. Sporulation dependent expression was observed in all three types. These results suggest that *cpeA* negative strains produce the factor or factors necessary for normal regulation of CPE-A expression. This could indicate that CPE-A expression is regulated by a common regulatory factor, such as a sigma factor, that also regulates other sporulation dependent gene expression.

It is unknown why sporulating cultures of *C. perfringens* produce such large amounts of the CPE-A. Originally, researchers believed CPE-A was a structural spore coat protein (McDonel, 1980b) or excess unincorporated spore coat protein (Labbe, 1980). Since less than 6% of strains carry the *cpeA* gene and *cpeA* negative strains can sporulate, it is unlikely CPE-A is an essential spore coat protein. Further work by Ryu and Labbe (1989) demonstrated that the CPE-A is not likely a major spore coat protein as originally thought, but that it can become trapped in developing spores during spore assembly and maturation. Multiple copies of the *cpeA* gene on the chromosome was another theory used to explain the copious amount of CPE-A produced upon sporulation. However, *cpeA* has been shown to exist only as a single copy on the chromosome (Canard *et al.*, 1992). Alternatively, CPE-A could be produced in large amounts by using some of the 10 putative RNA polymerase binding sequences identified by Brynestad *et al.* (1994) to increase levels of transcription. However, Melville *et al.* (1994) demonstrated that *cpeA* is transcribed from only one promoter 197 bp upstream of the translational start site. The long half life of the CPE-A mRNA could contribute to the large amounts of expressed CPE-A (Labbe and Duncan, 1977). The half-life of CPE-A mRNA is 58 minutes as compared to 2 or 3 minutes for most mRNA produced by *E. coli*.

Additional examination of the region 5' and 3' to the *cpeA* gene revealed conserved *Bacillus subtilis* Hpr consensus binding sequences both upstream and downstream of the gene (Brynestad *et al.*, 1994). The Hpr protein is a DNA binding protein that has been linked to the control of transition state functions in *B. subtilis* (Kallio *et al.*, 1991; Strauch and Hoch, 1993). The Hpr protein has a negative effect upon the expression of certain genes and most likely acts by binding to the promoter region and at a downstream site of the repressed gene forming a repression loop. Transcription of the *hpr* gene is positively regulated by the *B. subtilis* AbrB protein. Therefore, during exponential growth when AbrB levels are highest, so are Hpr levels, thereby preventing the expression of post-exponential specific genes. When exponential growth stops, AbrB synthesis is repressed. This in turn lowers the level of Hpr, thereby ending Hpr repressive effects.

Three Hpr binding sites have been identified upstream and one downstream of the *cpeA* gene (Brynestad *et al.*, 1994). Both the upstream and downstream Hpr binding sites are necessary for the full repressive effect of this transition state regulator. A probe homologous to a 586 bp fragment of the *B. subtilis hpr* gene hybridized to 9 *C. perfringens* strains, 6 of which carried the *cpeA* gene, suggesting *C. perfringens* contains a gene homologous to the *hpr* gene. Since CPE-A expression is linked to sporulation, the Hpr association could be of importance in determining how expression of the *cpeA* gene is controlled during sporulation.

CPE-A and Mobile Genetic Elements

Canard *et al.* (1992) determined that the *cpeA* gene maps to a highly polymorphic region of the chromosome. The *cpeA* gene occurs on a novel 5 Kb *Nru*I fragment that is situated in a 350 Kb interval between the ribosomal operons *rrnF* and *rrnG* called region "c". Region "c" is unique to *C. perfringens* strains that carry the *cpeA* gene. Therefore, only strains that carry the *cpeA* gene contain the novel 5 Kb *Nru*I fragment. This result, along with the finding that less than 6% of *C. perfringens* strains carry *cpeA,* is indicative of the gene being carried on a mobile genetic element.

Recently, *cpeA* was found on a large plasmid in certain strains (Cornillot *et al.*, 1995). In this study the majority (20 of 22) of the *cpeA* positive strains tested appeared to carry the gene on a large plasmid. These strains were of veterinary origin. The two strains that carried the *cpeA* gene on the chromosome were the cause of human foodborne illness. It is unknown how or why the *cpeA* gene became inserted into the chromosome of some *C. perfringens* strains if the plasmid was the origin of the enterotoxigenicity. Since the location of the *cpeA* gene seems to be site specific in the chromosome, a lysogenic bacteriophage may have integrated. Alternatively, region "c" could contain a hot spot for the insertion of a transposon.

To find clues to the control of expression of the CPE-A protein, Brynestad *et al.* (1994) sequenced 4.1 Kb of the region 5' to the *cpeA* gene. An open reading frame (ORF) with 66% DNA homology to the *Salmonella typhimurium* insertion sequence IS*200* was found approximately 1 Kb upstream of the *cpeA* gene. This ORF was conserved among all six *cpeA* positive strains of *C. perfringens* tested. Additionally, this ORF was found in the same position in all tested strains. It is unknown if a protein is expressed from this ORF; however, additional evidence points to this region being associated with mobilizable elements. First, this ORF does not contain any 6 base restriction enzyme palindromes which points to selection pressure from interspecies DNA transfer which could occur on mobile plasmids or bacteriophages. Second, sequences similar to the Tn*3* recombination site were observed both upstream and downstream of the *cpeA* gene. Therefore, the *cpeA* may be carried on a mobile genetic element. This is the case for other superantigenic enterotoxins, such as the *S. aureus* enterotoxins, where some, if not all, are phage mediated. It could be possible that superantigenic enterotoxins are of phage origin (Brynestad *et al.*, 1994).

C. perfringens Type A 8-6 Enterotoxin

In 1985 a unique *Clostridium perfringens* enterotoxin (CPE 8-6) was isolated from a mutant designated 8-6 (Lindsay *et al.*, 1985). The *C. perfringens* Type A 8-6 strain is a spontaneous mutant of strain NCTC 8798 first isolated by Duncan *et al.* (1972) during experiments that first linked enterotoxin production to sporulation. Lindsay *et al.* (1985) were operating under the assumption that the production of enterotoxin was linked to spore coat synthesis, which was later shown to be false. The 8-6 strain was studied because it was believed that a larger quantity of enterotoxin could be isolated from this strain since it produces spore coat proteins and heat stable spores, yet no spore coat was formed. However, instead of finding greater quantities of enterotoxin a unique *C. perfringens* enterotoxin, designated CPE 8-6, was discovered.

In genomic mapping experiments, Canard *et al.* (1992) found that *C. perfringens* type A 8-6 differed from the strain that it was derived from, NCTC 8798, by a 180 Kb insertion near the putative *terC* locus. This insertion is not within region "c" that contains the *cpeA* gene. Sporulation genes are located near the *terC* locus in *Bacillus subtilus*. Therefore, it has been suggested that the 180 Kb insert near the *terC* locus in strain 8-6 is responsible for the strain producing abnormal spores.

CPE 8-6 is similar to CPE-A in that it has a M_r of 36,000 based on polyacrylamide gel electrophoresis, while CPE-A has a molecular weight (MW) of 35,317 based on sequence analysis. CPE 8-6 differs from CPE-A both biochemically and in biological activity (Table 2-2). CPE 8-6 has been shown to have 2 subunits with isoelectric points of 3.8 and 4.3 (Lindsay *et al.*, 1985), while CPE-A is composed of a single polypeptide (Czeczulin *et al.*, 1993) with an isoelectric point of 4.3 (McDonel, 1980b). The first 40 N-terminal amino acids of CPE 8-6 have been sequenced (Martin, 1991). There is homology to amino acids 3 through 42 of the amino acid sequence of the CPE-A (Czeczulin *et al.*, 1993). However, the first two amino acids (Met and Leu) of CPE-A are not present in the CPE 8-6. N-terminal sequencing of CPE-A has shown that approximately half of CPE-A molecules lack the first Met residue (Richardson and Granum, 1983). This residue may be lost during post-translational processing, suggesting CPE 8-6 may also lose the first two amino acids to post-translational processing. CPE 8-6 is only slightly more stable than CPE-A as inactivation occurs at 10 minutes instead of 5 minutes at 60°C.

CPE 8-6 has a LD_{50} (median lethal dose) in mice intraperitoneally (i.p.) of 39.0 μg/kg, an erythemal unit (EU) of 0.10 μg (Niilo, 1975), and erythemal activity of 9800 EU/μg protein (Lindsay *et al.*, 1985). These values are three times greater than those observed for CPE-A, which has a mouse LD_{50} (i.p.) of 112.0 μg/kg, an EU of 0.29 μg, and erythemal activity of 3410 EU/μg protein (Niilo, 1975). Interestingly, CPE-A has been shown to be activated threefold when treated with trypsin (Richardson and Granum, 1983) or chymotrypsin (Granum and Richardson, 1991) to yield a biological activity similar to that of CPE 8-6. However, trypsinization of CPE 8-6 results in no increase in activity (Lindsay *et al.*, 1985).

CPE-A and CPE 8-6 are classified as enterotoxins because they cause fluid and electrolyte loss from the gastrointestinal tract. However, the 8-6 strain has never been implicated in human foodborne illness. The CPEs act in the

Table 2-2. Comparison of the physiochemical and biological characteristics of the *C. perfringens* type A and type A 8-6 enterotoxins.

Characteristic	Type A	Type A 8-6
Molecular mass	35,317[a]	36,000[b]
Subunit number	1	2
Isoelectric point	4.3	3.8 and 4.3
Heat stability (95% loss of activity)	5 min., 60°C	10 min., 60°C
Hydrophobicity index (KJ/residue)	4,133	4,079
Aggregation on SDS polyacrylamide gel electrophoresis	yes	yes
Mouse LD_{50} (ip) (μg/Kg)	112	39
One erythemal unit (EU) (μg)	0.29	0.10
Biological activation with trypsin	threefold	none

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[a] Based on amino acid sequence
[b] Based on SDS-PAGE analysis

Adapted from Lindsay *et al.*, 1985; McClane, 1994.

ileal region of the small intestine with decreasing activity as the intestine extends into the duodenum. However, CPE 8-6 causes greater damage *in vivo* to intestinal tissue than does CPE-A. In the rat and rabbit ileum CPE-A acts at the villus tip causing desquamation of epithelial cells and the formation of membrane blebs; however, there is no damage to the lamina propria (McDonel *et al.*, 1978). In contrast, CPE 8-6 can cause damage to the lamina propria in rabbit (Lindsay and Dennison, 1986a) and mouse (Lindsay and Dennison, 1986b) ileal tissue, along with varying degrees of damage to the villus enterocytes. Lindsay (1988b) found that CPE 8-6 inhibits DNA, RNA, and protein synthesis, and causes the reversal of glucose transport as does CPE-A (McClane, 1994). Therefore, the mechanism of action of CPE 8-6 may be similar to that of CPE-A.

The action of CPE-A and CPE 8-6 can be activated by the addition of a molecule that is present in the brush border membrane (BBM) isolated from young rabbits (Mach and Lindsay, 1994). The activator molecule is interferon-gamma (IFN-γ) (Mach and Lindsay, unpublished observations) which may enhance cell permeability, allowing a more rapid uptake of toxins and cell death at lower concentrations of enterotoxin. CPE 8-6 remains more biologically active than CPE-A in the presence of IFN-γ (Table 2-3).

Despite this evidence, some researchers do not agree with the existence of a second type of *Clostridium perfringens* enterotoxin (Rood and Cole, 1991; Stelma *et al.*, 1985). They believe *C. perfringens* type A 8-6 produces the same enterotoxin as all other enterotoxin producing type A strains. One group has recently sequenced the *cpe86* gene from a *C. perfringens* type A 8-6 (Cornillot *et al.*, 1995). The sequence was completely identical with the previously published *C. perfringens* type A NCTC 8239 (Czeczulin *et al.*, 1993). It is unclear if different *C. perfringens* type A 8-6 strains exist.

Table 2-3. The effect of CPE 8-6 and CPE-A on Vero cell death *in vitro* after treatment with IFN-γ.

	% Cell Death	
Treatment	IFN-γ[a]	IFN-γ[b]
0.8 ng CPE 8-6	83 ± 6	28 ± 7
3.0 ng CPE-A	85 ± 5	29± 6

[a] Pretreatment with 50,000 units of IFN-γ for 4 hours, removed, and treated with CPE.

[b] IFN-γ incubated with CPE for 1 hour before addition to cells.

It is possible that the *C. perfringens* enterotoxins (CPE-A and CPE 8-6) are related to each other as the *Staphylococcus aureus* enterotoxins (SEA, SEB, SEC, SED, and SEE) are related. The *S. aureus* enterotoxins (SE) cause foodborne illness by intoxication, not infection (Betley and Harris, 1994; Salyers and Whitt, 1994). The incubation period ranges from 30 minutes to 8 hours and the symptoms are abdominal pain and nausea, followed by vomiting and diarrhea. SEs are 30 kDa superantigenic proteins that have considerable amino acid and nucleotide sequence homology and are differentiated on the basis of their antigenic properties. The different types of *S. aureus* enterotoxins are produced in different amounts by the strains that carry them, and the genes encoding them have been found on the chromosome, plasmids and temperate bacteriophage suggesting their association with a mobile genetic element. The CPEs are similar in size to the SEs and the CPEs are antigenically different from each other as are the SEs (Lindsay *et al.*, 1985). Both CPEs have been found to be superantigenic and can out-compete staphylococcal enterotoxin A for binding to class II major histocompatability complex molecules (Bowness *et al.*, 1992; Lindsay and Johnson, unpublished observations). The *cpeA* gene has been found both in chromosomal and plasmid locations (Cornillot *et al.*, 1995) as have the SEs.

Cloning and characterization of the *C. perfringens* type A 8-6 enterotoxin gene will determine if there are indeed two *C. perfringens* enterotoxin genes and how they differ. This work could also provide information about the conserved and variable regions of the *C. perfringens* enterotoxin amino acid and nucleotide sequence. The CPE 8-6 is more biologically active than the CPE-A. Thus, by comparing the sequences of the two proteins, regions responsible for specific activities of the enterotoxins could be identified. This information could provide a basis for future research on the mechanisms of pathogenicity and regulation of virulence expression in *C. perfringens*.

CHAPTER 3
COMPARATIVE SEROLOGICAL, BIOLOGICAL, AND RESTRICTION ENZYME ANALYSIS OF *CLOSTRIDIUM PERFRINGENS* TYPE A STRAINS

Introduction

There is considerable evidence indicating that some *C. perfringens* type A strains produce a unique enterotoxin, designated CPE 8-6, that expresses different biological and biochemical activities when compared to the better characterized type A enterotoxin, CPE-A. Despite this evidence, there is still a great deal of controversy with respect to the existence of a second enterotoxin. The overall goal of this dissertation is to confirm using current molecular biology techniques that two toxins do indeed exist. The aims of the work described in this chapter are to screen *C. perfringens* strains to determine enterotoxigenic activity, to identify any differences in restriction enzyme profiles between strains using a series of DNA probes, and to determine the location of the *cpe* genes when present.

Materials and Methods

Bacterial Strains

The *C. perfringens* type A 8-6 strains used were NCTC 8798 8-6M, 8-6S, and 86-11, obtained from Dr. James Lindsay, University of Florida, Gainesville, FL. The cultures were initially obtained from Dr. Madeline Sebald, Pasteur Institute, Paris, France, who first isolated and characterized the 8-6 type (Duncan *et al.*, 1972). Strain 8-6M has been previously described by Martin

(1991) as an enterotoxigenic 8-6 strain. Strain 8-6S had been stored as freeze dried spores generated when the CPE 8-6 toxin was produced and characterized (Lindsay *et al.*, 1985). Strain 86-11 is a culture of 8-6S grown and stored as vegetative cells in pork infusion agar. Type A strain NCTC 8239 is the well characterized CPE-A enterotoxin producer (Czeczulin *et al.*, 1993). NCTC 3624 is a non-enterotoxigenic type A strain (Czeczulin *et al.*, 1993). Both 8239 and 3624 were kindly provided by Dr. Bruce McClane, University of Pittsburgh, Pittsburgh, PA. All *C. perfringens* cultures used in this study were maintained as spore suspensions in cooked meat media (Difco Laboratories, Detroit, MI) frozen at -20°C.

Escherichia coli ATCC 11303 and *Klebsiella oxytoca* M5A1 genomic DNA was kindly provided by Dr. Lonnie Ingram, University of Florida. *Bacillus subtilis* (Lindsay, 1988a) and *Listeria monocytogenes* ATCC 15313 (Van Der Kelen and Lindsay, 1991) were obtained from Dr. James Lindsay and grown as described previously. *Vibrio cholerae* enterotoxigenic strain 401 was kindly provided by Dr. Mark Tamplin, University of Florida, and was grown on heart infusion agar (Difco Laboratories). *Campylobacter jejuni* ATCC 29428, *Enterococcus faecalis* ATCC 19633, *Salmonella enteritidis* ATCC 13076, and *Shigella sonnei* ATCC 25931 were all grown on Luria Broth (LB) agar (Luria and Delbruck, 1943). *Yersinia enterocolitica* ATCC 23715 was grown on Yersinia Selective Medium (Oxoid, Basingstoke, UK).

Preparation of Antisera to CPE-A and CPE 8-6

Purified CPE-A was obtained from Dr. Bruce McClane, University of Pittsburgh, and purified CPE 8-6 from Dr. James Lindsay, University of Florida. Antisera to the *C. perfringens* CPE-A and CPE 8-6 enterotoxin proteins were prepared by Kelfarms, Alachua, FL. Three mg of each purified enterotoxin were supplied as the antigens. Individual 4-5 Kg white rabbits were used to generate each antisera. One milliliter of Freund's Complete adjuvant was emulsified with 1 mg (300 µl) of the antigen and 5 injections (3 intradermal, 1 subcutaneous, 1 intramuscular) were given to the rabbit. These injections were repeated using 1 mg of antigen and 1 ml of Freund's Incomplete adjuvant 30 days later and again 14 days after that. The titre was monitored by test bleeds from the ear vein every 7 days after an injection. After the third series of injections, the rabbit was anesthetized with Ketamine and Rompun and bled by cardiac puncture. The serum was separated by centrifugation and stored at -20°C.

Biotinylation of Antisera

Antisera to the *C. perfringens* type A (8-6) and the 8239 enterotoxins were biotinylated using NHS-LC-Biotin (Pierce, Rockford, IL). One hundred microliters of 0.05 M bicarbonate buffer, pH 8.5, was added to 2 mg of each antisera. NHS-LC-biotin (0.04 mg) was added to the antisera and incubated on ice with mixing every 20 minutes for 2 hours. Unreacted biotin was removed by adding phosphate buffered saline (PBS) to a total volume of 1 ml and dialyzing in 6,000 to 8,000 molecular weight cut off dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) against two 500 ml volumes of PBS overnight. The biotin labeled antisera was aliquoted and stored at -20°C.

Enterotoxin Production

C. perfringens strains were examined for enterotoxin production by enzyme linked immunosorbent assay (ELISA) after growth and sporulation in either cooked meat media (CMM: Difco Laboratories) or modified Duncan Strong media (MDS) (yeast extract, 0.4%; sodium thioglycolate, 0.1%; raffinose, 0.4%; proteose peptone, 1.5%; sodium phosphate dibasic, 0.52%; Duncan and Strong, 1968).

For growth and sporulation in CMM, 50 µl of stock spore suspension was inoculated into 15 ml of fresh CMM, heated at 75°C for 10 minutes, cooled, and incubated at 37°C for 36 hours. Cultures were examined for spores, and were used if sporulation was greater than 25%. For growth and sporulation in MDS, 50 µl of stock spore suspension was inoculated into 10 ml of 3 day old fluid thioglycolate (FT: Difco Laboratories), heated at 75°C for 10 minutes, cooled and incubated at 37°C for 18 hours. One milliliter of this culture was transferred to 10 ml of MDS and incubated at 37°C for 16 hours. Cultures were examined for spores and were used if sporulation was greater than 25%. For the ELISA 1 ml volumes of both CMM and MDS cultures were centrifuged at 12,000 x g for 10 minutes and the supernatant collected. The pelleted spores were inoculated into trypticase sulfate neomycin agar (TSN: Difco Laboratories) and incubated anaerobically at 37°C to verify purity.

Larger volumes of sporulating clostridial cultures in MDS were also prepared for ELISA, Western immunoblot, and Vero cell cytotoxicity assays. Ten milliliters of FT culture were prepared as described above and inoculated into 1 L of MDS and incubated for 16 hours at 37°C. To collect the protein, the cultures were centrifuged at 10,000 x g for 20 minutes and the spores discarded. One molar $MnCl_2$ was added to the supernatant to give a final concentration of 0.05 M $MnCl_2$ in order to precipitate all nucleic acids, which were then removed by centrifugation at 10,000 x g for 20 minutes (Lindsay *et al.*, 1985). Ammonium sulfate was slowly added to the supernatant to 80% saturation and was left stirring at 4°C for 16 hours. The solution was centrifuged at 10,000 x g for 30 minutes, the supernatant was discarded, and the precipitate resuspended in approximately 5 ml of 10 mM Tris/HCl, 1 mM EDTA, pH 7.0

(TE). This solution was dialyzed in 6,000 to 8,000 molecular weight (MW) cut off dialysis tubing (Spectrum Medical Industries, Inc.) against 3 changes of 1 L of TE buffer over 16 hours. After dialysis the protein solution was removed from the tubing and centrifuged at 10,000 x g for 10 minutes to remove any denatured protein. The proteins were concentrated to 2 ml using an Amicon (Beverly, MA) Centricon concentrator with a 10,000 MW cut off and stored frozen at -20°C.

The amount of protein present in each sample was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin used as the standard.

Enzyme Linked Immunoassay

According to Seymour *et al.* (1991) 50 µl of sample, either supernatant from growth in MDS or CMM, or concentrated culture supernatant, and 50 µl of coating buffer (1.5 mM Na_2CO_3, 3.3 mM $NaHCO_3$, 3 mM NaN_3) were added to individual wells of a polystyrene 96 well Immulon™ 2 flat bottom plate (Dynatech Laboratories, Chantilly, VA), and incubated overnight at 4°C. This solution was aspirated and washed 4 times with 100 µl of phosphate buffered saline and Tween (10 mM $NaHPO_4$, 10 mM Na_2HPO_4, 0.15 M NaCl, and 0.2% Tween 20, pH 7.2; PBS:TW). One hundred microliters of primary polyclonal antibody to CPE-A or CPE 8-6 was diluted to 10^{-3} in PBS:TW, added to each well and incubated at room temperature for 2 hours. The wells were aspirated and washed 4 times with 100 µl of PBS:TW. One hundred microliters of the secondary antibody, goat-anti rabbit IgG (Sigma Chemical Co., St. Louis, MO), diluted 10^{-3} in PBS:TW, was added to each well and incubated at room temperature for 2 hours. The wells were again aspirated and washed as before. The antigen:antibody complex was detected by the addition of 100 µl of alkaline phosphatase buffer (50 mM Na_2CO_3, 50 mM $NaCO_3$, 0.5 mM $MgCl_2$)

containing 100 μg of p-nitrophenyl phosphate (Sigma Chemical Co.). The positive alkaline phosphatase reaction results in a yellow color, which is stopped after 4 hours by the addition of 100 μl 0.1 N NaOH. The reaction was analyzed spectrophotometrically at 405 nm using a Bio-Rad Model 2550 ELISA reader.

Western Immunoblot

Proteins were separated by polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulfate (SDS) using Bio-Rad Mini-Protean II slab gels and the buffer system of Laemmli (1970). Stacking and separating gels were 4.0% and 13.5% polyacrylamide, respectively. Molecular weight standards (14.4 to 97.4 kDa) were obtained from Promega Corp. (Madison, WI). Gels were stained with 0.15% Coomassie Brilliant Blue R-250 for 1 hour or used for Western immunoblots. For Western immunoblots the gel was soaked in transfer buffer (0.192 M glycine, 0.025 M Tris, 20% methanol) for 20 minutes. Western immunoblotting was accomplished using the Bio-Rad Mini Trans-Blot electrophoretic transfer cell and nitrocellulose membranes (Stratagene, La Jolla, CA) according to manufacturers directions. After transfer, the membrane was blocked with PBS containing 0.1% Tween 20 and 7.0% casein for 1 hour. The membrane was drained and soaked overnight in a 10^{-3} dilution of biotinylated antisera in blocking buffer. The membrane was then washed and soaked in a final concentration of 1 mg/ml strepavidin (Sigma Chemical Co.) for 2 hours. After a second washing step the blot was soaked with substrate buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) 3 times for 5 minutes each. Finally, the membrane was soaked with 0.1 mg/ml of nitroblue tetrazolium and 0.05 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in substrate buffer until bands appeared.

Vero Cell Cytotoxicity Assay

African Green Monkey kidney cells (Vero) (ATCC CCL 81) were cultured in Sarstedt (Newton, NC) 75 cm^2 flasks and maintained in modified Eagles medium (MEM) containing 10% fetal bovine serum (FBS). Cells were incubated in moist 5% CO_2/95% air at 37°C. When monolayers reached confluency (10^5 cells/ml) they were removed with trypsin-EDTA, diluted to 20 ml in MEM with 10% FBS, and 400 μl was added per well to Corning 24 well cluster dishes. Plates were incubated for 24 hours, the medium was removed, and the cells washed twice with 15 mM PBS. Cells were then incubated in 300 μl of M199 media (FBS free). To test the cytotoxicity of CPE-A, CPE 8-6, and cellular lysates of clones, the proteins were added to the Vero cells in cluster dishes (total well volume 400 μl) and incubated for up to 90 minutes. After incubation the M199 and stimulant was removed from each well, and 0.04% trypan blue in PBS was added. Cells were examined for viability and morphological damage with a Nikon phase contrast microscope.

Preparation of DNA For Dot Blots

Genomic DNA from *V. cholerae*, *C. jejuni*, *E. faecalis*, *S. enteritidis, S. sonnei*, *B. subtilis*, *Y. enterocolitica*, and *L. monocytogenes* was isolated by scraping colonies off of plates and resuspending the growth in TE, pH 8. One mg of lysozyme (Sigma Chemical Co.) and 50 ml of 10% SDS solution was added and incubated overnight at 37°C overnight. Cellular debris was removed by centrifugation at 10,000 x g for 10 minutes and the supernatant was saved as the DNA containing fraction. *C. perfringens* genomic DNA was prepared as described in the following Preparation of *C. perfringens* Genomic DNA section.

Approximately 30 μg of DNA was added to 0.5 μg of dot blot denaturing solution (4 M NaOH, 100 mM EDTA). Five to ten micrograms of genomic DNA was dotted onto nylon membranes, UV cross linked, and prehybridized. The membranes were hybridized with probes 1-4 at 60°C as described in the Southern Detection section. Detection was accomplished with the Boehringer Mannheim Genius™ kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Preparation of *C. perfringens* Genomic DNA

For the preparation of *C. perfringens* genomic DNA, 50 μl of the stock CMM culture was inoculated into 10 ml of fluid thioglycolate, heated to 75°C for 10 minutes and incubated at 37°C for 18 hours. One milliliter of these cultures was inoculated into 1 L of MDS and incubated at 37°C for 8 hours. The cells were collected following centrifugation at 10,000 x g for 15 minutes, washed with 10 mM EDTA, 25 mM Tris-HCl, 10% glycerol and centrifuged again (Byun *et al.*, 1986). The supernatant was removed and the cells resuspended in 10 mM EDTA, 25 mM Tris-HCl, 10% glycerol using a volume that gave a concentration of 100 mg wet weight cells per ml. To this cell suspension 1 mg of lysozyme/ml (Sigma Chemical Co.) was added, followed by incubation for 18 hours at 37°C. Proteinase K (Promega Corp.) was added to a concentration of 200 μg/ml followed by incubation at 50°C for 12 hours. Then 100 μl of 10% sodium N-laurylsarcosinate/ml of reaction mixture was added and incubated at 50°C for 18 hours. Two volumes of ice cold 95% ethanol were added to the DNA. The DNA was spooled with a Pasteur pipette, rinsed with 70% ethanol and dried at 37°C for 1 hour. The DNA precipitate was dissolved in a 50 ml sterile screw cap tube in 5 ml of 10 mM Tris-HCl, 1 mM EDTA, pH 7 by gently rocking the tube for 4 hours. The solution was treated with DNase free RNase (Boehringer Mannheim Biochemicals) for 30 minutes at 37°C. A 25:24:1

buffered phenol/chloroform/isoamyl alcohol extraction (Sigma Chemical Co.) was performed to purify the DNA. One milliliter of phenol/chloroform/isoamyl alcohol was added to 1 ml of the DNA solution and gently mixed for 30 seconds. The solution was then centrifuged for 15 seconds at 12,000 x g at room temperature in a microcentrifuge. The aqueous phase containing the DNA was transferred to a new tube. The phenol/chloroform/isoamyl alcohol step was repeated until there was no white precipitate present at the aqueous/organic interface. The residual phenol/chloroform/isoamyl alcohol was removed by ethanol precipitation as described by Ausubel *et al.* (1989).

Restriction Enzyme Analysis

Genomic DNA from *C. perfringens* strains 8239, 8-6M, 8-6S, and 86-11 was digested according to manufacturer's specifications with the restriction enzymes *Sau*3A, *Mbo*I, *Eco*RI, *Pst*I, *Hin*dIII, *Xba*I, *Bam*HI and *Nru*I (Promega Corp.). The digests were separated by agarose gel electrophoresis for 1 hour at 75 V using a Bio-Rad (Melville, NY) Mini Sub Cell. The gels were 0.8% agarose in 0.04 M Tris-acetate, 1.0 mM EDTA, pH 8.0 (TAE buffer) containing 1 µg/ml of ethidium bromide. SPP1-DNA digested with *Eco*RI (Boehringer Mannheim Biochemicals) and a *Hin*dIII digest of bacteriophage λ (Promega Corp.) were used as molecular weight markers.

DNA Oligonucleotide Construction and Labeling

Five oligonucleotides representing different regions of the 8239 *cpeA* gene (Van Damme-Jongsten *et al.*, 1990) were synthesized by the DNA Synthesis Core Laboratory at the University of Florida using an Applied Biosystems 380B synthesizer (Figure 3-1). Probe 1 consisted of a nucleotide

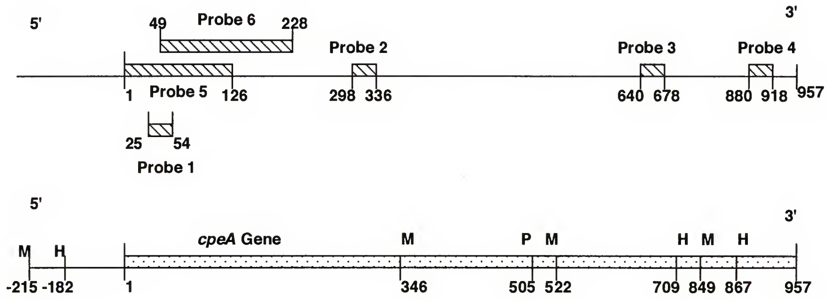


Figure 3-1 Physical map of *C. perfringens* type A NCTC 8239 *cpeA* gene. The *cpeA* gene is indicated by the stippled rectangle. Restriction enzyme sites are shown for *Mbo* I (M), *Hin*dIII (H), and *Pst* I (P). The positions of the DNA probes are indicated by the hatched rectangles.

sequence from base pairs 25 to 54 (5'-ATGGTGTTCGAAAACGCTAAAGAAGTATTT-3'); probe 2 from base pairs 298 to 336 (5'-GTAGAATATGGATTTGGAATAACTATAGGAGAAGAAAAT-3'); probe 3 from base pairs 640 to 678 (5'-GCATTAAACTCAAACCCAGCTGGAAATTTATATGATTGG-3'); and probe 4 from base pairs 880 to 918 (5'-GGACAATATGTTCTTGTAATGAAAGCTAATTCATCATAT-3').

Only recently it was discovered that the published *cpeA* nucleotide sequence used to design these probes was not that of the 8239 strain as originally reported (Van Damme-Jongsten *et al.*, 1989). The *cpeA* sequence was actually from strain F3686 (Cornillot *et al.*, 1995). Czeczulin *et al.* (1993) discovered the error and showed that there were eight nucleotide base differences between the two ORF sequences. Seven of these changes were silent at the amino acid level and one of the differences at nucleotide 331 changes the GLU amino acid of the F3686 to a GLN amino acid in the 8239 *cpeA*. The 8239 *cpeA* sequence has a TAC nucleotide sequence for the codon specifying amino acid 310 of CPE-A, while the F3686 has 2 codons (TCT CAC) at this location. Therefore, probes 1 through 3 are actually based on the F3686 *cpeA* gene which is slightly different from that of the 8239 *cpeA* gene. An 8239 *cpeA* specific probe would require changing nucleotide 39 of probe 1 from a C to a T, nucleotide 331 of probe 2 from a G to a C, nucleotide 654 of probe 3 from a C to a T, and nucleotide 663 of probe 3 from an A to a T. The nucleotides involved in the changes are underlined above. In the region of probe 4 there were no differences between the sequence for the 8239 and the F3686 *cpeA* gene.

Probe 5 consisted of nucleotides 1 through 125 of the *cpeA* as determined by Czeczulin *et al.* (1993) (Figure 2-2). Probe 6 was a 180 bp DNA fragment synthesized by the polymerase chain reaction (PCR). The primers were designed according to the published sequence of the CPE-A gene (Czeczulin *et al.* 1993). Primer 1 was the forward primer and consisted of nucleotides (5') 49 to 70 (3') of the CPE-A gene. Primer 2 was the reverse primer and consisted of nucleotides (5') 228 to 207 (3'). These primers were also synthesized by the DNA Synthesis Core Laboratory at the University of Florida. They were used to amplify the 180 bp fragment of DNA from the NCTC 8239 strain. The amplification reaction mixture consisted of 10 μl of 10x PCR buffer (Promega Corp.), 8 μl 25 mM $MgCl_2$, 4 μl each of primer 1 and 2 diluted to 10 mM, 10 μl of 2 mM dNTP mix, 5 μl of template DNA at 100 μg/ml, 0.25 μl of *Taq* polymerase (Promega Corp.), and 30.75 μl of water. The cycling parameters were 94°C for 4 minutes, then 30 cycles of 94°C for 1 minute, 42°C for 1 minute, and 72°C for 1 minute, followed by one cycle of 72°C for 7 minutes. The reaction was done using a SingleBlock™ EasyCycler™ series thermal cycler (Ericomp, Inc., San Diego, CA). The PCR product was subjected to agarose gel electrophoresis, the 180 bp product was cut out of the gel with a razor blade and purified using the Qiaex (Chatsworth, CA) Gel Extraction kit according to manufacturers directions.

Oligonucleotide Labeling

All of the oligonucleotides were non-radioactively labeled by 3' tailing with digoxigenin 11-dUTP/dATP (Boehringer Mannheim Biochemicals) using terminal transferase (Boehringer Mannheim Biochemicals) as recommended by the manufacturer, to give a final concentration of labeled probe of 600 ng/ml. The probes were stored at -20°C.

Southern Analysis

After electrophoresis of restriction digested DNA, the agarose gels were denatured in 0.5 N NaOH, 1.5 M NaCl for 1 hour and then neutralized in 1.0 M Tris-HCl, pH 8.0; 1.5 M NaCl for 1 hour. The DNA was then blotted from the gel to nitrocellulose membranes (Stratagene) using a Stratagene PosiBlot pressure blotter and UV crosslinked to the membranes using a Stratagene Stratalinker 1800. Southern blot hybridizations were performed as recommended by the Genius™ Nonradioactive DNA Detection Kit (Boehringer Mannheim Biochemicals). The UV crosslinked membranes were prehybridized in 40 ml of prehybridization solution in a pint-sized Dow heavy duty Ziploc freezer bag submerged in a shaking water bath (35 rpm) at 42°C or 47° for 1 hour. The prehybridization solution was discarded and the prehybridized membranes and hybridization solution were sealed in the same bag and submerged in a shaking water bath at 42°C or 47°C overnight. Detection of the hybridized probe was accomplished by colorimetric detection with the Boehringer Mannheim Genius™ kit. Detection employed an anti-digoxigenin antibody conjugated to alkaline phosphatase. The antibody conjugate was visualized using the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride.

PCR Analysis

Four sets of PCR primers were synthesized in order to probe the genomes of strains 86-11, 8-6S, and 8-6M for any nucleotide sequences that resembled those the *cpeA* gene. The PCR primers were synthesized based on the published 8239 *cpeA* sequence (Czeczulin *et al.*, 1993) and the published promoter sequence from NCTC 8798 and NCTC 10240 (Melville *et al.*, 1994) and used as described in the Oligonucleotide Construction and Labeling section. The nucleotide sequence and location of the PCR primers is shown in Table 3-1 and the expected amplified fragment sizes are shown in Table 3-2.

Isolation of and Characterization of Plasmids From *C. perfringens*

Plasmid DNA from 8239, 86-11, 8-6S, and 8-6M was isolated according to Roberts *et al.* (1986). The isolated DNA was subjected to agarose gel electrophoresis, blotted to a nitrocellulose membrane, and detected with probe 6 as previously described.

Results and Discussion

Enterotoxigenic strains of *C. perfringens* only produce large amounts of enterotoxin during sporulation. If an enterotoxigenic strain does not sporulate, enterotoxin is not produced in large amounts, and assays for the detection of enterotoxin may yield negative results. Uniform and reproducible sporulation can be difficult to achieve in the laboratory. However, sporulation usually occurs in MDS and CMM media. MDS was developed especially to force sporulation of *C. perfringens* in the laboratory, and contains raffinose as the limiting carbohydrate source. Sporulation usually occurs in CMM although not at as high a level as occurs in MDS. CMM is a medium generally used for the preparation of stock cultures.

Enzyme linked immunosorbent assays (ELISA) were completed on the supernatants of sporulating *C. perfringens* cultures grown in CMM and MDS. Antisera to both the CPE-A and CPE 8-6 were used (Table 3-3). All tested strains (8239, 86-11, 8-6M, and 8-6S) produced enterotoxin in MDS and CMM, and reacted with antisera to both CPE-A and CPE 8-6. The strains produced more enterotoxin when cultured in MDS than when cultured in CMM.

Table 3-1. Nucleotide sequence of PCR primers.

Primer number	Region of sequence	Nucleotide sequence
1	*cpeA* gene nt 49 to 70	5'-GTATTTCTTATTTCTGAGGAT-3'
2	*cpeA* gene nt 207 to 228	5'-AGTTAATGATTGGCTAAAGGTA-3'
3	*cpeA* gene nt 339 to 359	5'-CACATCTTTCACCAGTTTCAA-3'
4	*cpeA* gene nt 871 to 892	5'-GAACATATTGTCCAGCATCTAA-3'
5	*cpeA* promoter nt 122 to 141	5'-ATAATATATCACTCTCCTTG-3'
6	*cpeA* gene nt 111 to 130	5'-TTACATATAATCCATCACTT-3'

The *cpeA* gene and promoter nucleotide sequences are shown in Figures 2-2 and 2-3 respectively. The nucleotide (nt) numbers given above refer to these Figures.

Table 3-2. Expected size of PCR fragments.

Primer Set	Primer Pairs	Expected Amplified Fragment Size
A	1 and 2	180 bp
B	1 and 3	510 bp
C	1 and 4	843 bp
D	5 and 6	446 or 491 bp

Table 3-3. ELISA Results.

Supernatant from cooked meat media (ng of enterotoxin/sample).

Strain	Antisera to CPE-A	Antisera to CPE 8-6
8239	8.5	3.7
8-6M	4.0	1.6
86-11	6.8	3.6
8-6S	7.2	3.9

Supernatant from modified Duncan Strong media (ng of enterotoxin/sample).

Strain	Antisera to CPE-A	Antisera to CPE 8-6
8239	>10.0	>10.0
8-6M	4.0	2.2
86-11	>10.0	>10.0
8-6S	>10.0	>10.0

In MDS strains 8239, 86-11, and 8-6S produced greater than 10 ng/sample of enterotoxin as detected by both the antisera to CPE-A and to CPE 8-6. Since the optimal antisera titre (1:1000) was used, the actual amount produced can only be estimated at 40 ng of enterotoxin for each strain. The enterotoxin produced by strain 8239 cross reacted with the antisera to CPE 8-6, and the enterotoxin produced by the 8-6 strains (86-11, 8-6M, and 8-6S) cross reacted with the antisera to CPE-A suggesting similarities between the CPE-A and CPE 8-6.

Strain 8-6M has been previously described by Martin (1991) as an enterotoxigenic 8-6 strain. Strain 8-6S had been stored as freeze dried spores generated when the CPE 8-6 toxin was produced and characterized (Lindsay *et al.*, 1985). Strain 86-11 is a culture of 8-6S grown and stored as vegetative cells in pork infusion agar. Type A strain NCTC 8239 is the well characterized CPE-A enterotoxin producer (Czeczulin *et al.*, 1993). Strains 86-11 and 8-6S produced enterotoxin at a level similar to that of the 8239 strain. Strain 8-6M produced approximately 2 to 3 fold less enterotoxin than the 8239, 86-11, and 8-6S strains. This could suggest differences between 8-6M and the other 8-6 strains since sporulation and enterotoxin production is strain dependent.

Only recently, *C. perfringens* ATCC 3624 was kindly provided by Dr. Bruce McClane, University of Pittsburgh. This strain does not contain the enterotoxin gene. A partially purified preparation of the supernatant was prepared from this strain as described in the Enterotoxin Production section. ELISAs were repeated using the partially purified supernatants from 3624, 8239, 86-11, and 8-6M (Table 3-4).

These ELISA results suggest that all of the tested strains produce some enterotoxin, including 3624 which does not carry the *cpeA* gene (Czeczulin *et al.*, 1993). Therefore, it was concluded that the ELISA produced false positive

Table 3-4. Recent ELISA Results.

Partially purified supernatant from modified Duncan Strong media (ng of enterotoxin/sample).

Strain	Antisera to CPE-A	Antisera to CPE 8-6
8239	5.6	4.8
8-6M	3.6	1.5
86-11	7.5	5.7
3624	3.7	1.3

results. This has been previously observed by Van Damme-Jonsten *et al.* (1990) and Berry *et al.* (1988). The ELISA could be reacting to a 48 kDa spore coat protein that was observed by Ryu and Labbe (1989 and 1993) to crossreact with antisera to *cpeA*. This 48 kDa protein was observed in these studies to be present in the 3624 strain. Ryu and Labbe suggest this 48 kDa protein could be a precursor to mature CPE-A that is only processed to the mature 35 kDa CPE-A in certain strains. However, Czeczulin *et al.* (1993) observed that mature CPE-A contains 319 amino acids which match the ORF predicted sequence of *cpeA* and in frame termination codons immediately preceding and following the ORF of the cloned *cpeA* gene. Recombinant CPE-A made in *E. coli* comigrates with native mature CPE-A in SDS-PAGE analysis. All of these points argue against the presence of a CPE-A precursor molecule. However, this 48 kDa protein does cross-react with *cpeA* antisera and is present in *C. perfringens* strains that have been shown not to carry the *cpeA* gene. It may be possible that this spore coat protein is not related to the CPE-A, but has a common epitope that the anti-CPE-A recognizes. If this were true, serological assays like the ELISA, which don't discriminate the size of the molecule that reacts with the antisera, may produce false positive results. An assay that reveals the size of the reacting molecule, like the Western immunoblot, could discriminate between a positive 35 kDa band that is most likely the CPE-A and a positive 48 kDa band.

Since the ELISA assay has been shown to produce false positive and false negative results (Van Damme-Jonsten *et al.*, 1990; Berry *et al.*, 1988), the Western immunoblot was used to examine the *C. perfringens* strains for enterotoxin production. Kokai-Kun *et al.* (1994) found that Western immunoblots were specific for the presence of the CPE-A. The partially purified and concentrated supernatants were subjected to electrophoresis using SDS-

PAGE and blotted to nitrocellulose for Western analysis using antisera to the CPE 8-6 (Figure 3-2). Western analysis revealed a 35 kDa protein that reacted with the CPE 8-6 antisera in the 8239 preparation. The 86-11 preparation had a 35 kDa protein that only slightly reacted with the CPE 8-6 antisera. The 3624 and 8-6M preparations did not have any 35 kDa proteins react with the antisera. However, there were heavier positive bands in all preparations which would support the positive ELISA results in the absence of enterotoxin. The positive and negative controls functioned correctly in each of these experiments. Strain 8-6M gave a negative result in the Western immunoblot assay, providing additional evidence that the ELISA produced a false positive result with this sample both in this study and in the study by Martin (1991).

Protein from the supernatant of the sporulating cultures was isolated, purified, and concentrated. One hundred microliters of the concentrated supernatant was added to the Vero cells to determine the cytotoxicity of the preparation. The protein from strains 8239 and 86-11 was cytotoxic to the Vero cells, while the protein from strains 8-6M and 3624 was not (Figure 3-3).

The key to these enterotoxin detection experiments was to achieve sporulation of the strains so they would produce the enterotoxin. Strain 8239 had sporulation rates of 40-60%, strain 86-11 had rates of 10%, strain 8-6M had rates of 50-90%, and strain 3624 had rates of 30-40%. Strain 8-6M gives contradictory results in the detection assays. This strain sporulated the best out of all the strains (50-90%) and exhibited a positive ELISA result, but did not exhibit Vero cell cytotoxicity or a 35 kDa band in Western blotting. Strain 86-11 sporulated poorly (10%), but still produced enough enterotoxin to produce a positive ELISA result, a positive Vero cell cytotoxicity result, and a slight positive Western reaction. Therefore, poor sporulation could not be attributed as the cause of poor enterotoxin production by strain 8-6M in the Western immunoblot

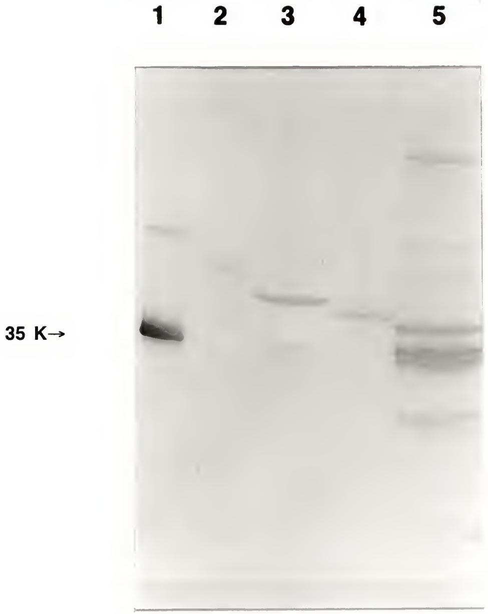


Figure 3-2. SDS-PAGE and Western analysis of purified and concentrated supernatants of 8239, 3624, 86-11, and 8-6M detected with a 10^{-3} dilution of antisera to CPE 8-6. The amount of protein in each sample is noted in parenthesis. Lane 1: purified CPE 8-6 (0.5 μg); lane 2: 3624 supernatant (100 μg); lane 3: 86-11 supernatant (91 μg); lane 4: 8-6M supernatant (30 μg); lane 5: 8239 supernatant (91 μg).

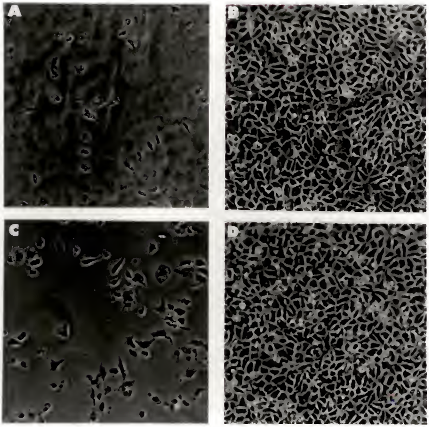


Figure 3-3. Vero cells treated with purified supernatant fractions of sporulating 8239, 3624, 86-11, and 8-6M cultures. Vero cells treated with: (A) 8239 supernatant; (B) 3624 supernatant; (C) 86-11 supernatant; (D) 8-6M supernatant.

and Vero cell cytotoxicity assays. However, strain 3624, a proven *cpeA* negative strain, also produces a positive ELISA result suggesting the 8-6M positive ELISA result is a false positive.

The 8-6S and 86-11 strains produced similar results in all assays. The ELISA, Western immunoblot, and Vero cell cytotoxicity assays suggest that the 8-6 strains fall into two Groups (Table 3-5). Group 1 consists of strains 8-6S and 86-11 which produce positive ELISA results that are 2-3 fold higher than the Group 2 positive ELISA results and positive Western immunoblot and Vero cell cytotoxicity results. Strain 86-11 was used as the representative strain of Group 1. Group 2 consists of strain 8-6M that produces positive ELISA results, negative Western immunoblot results, and negative Vero cell cytotoxicity results.

To further evaluate the enterotoxigenicity of these strains six probes were synthesized. DNA probes only test for the potential of *C. perfringens* strains to produce enterotoxin since the presence of the expressed protein is not demonstrated. To evaluate the efficacy of the probes and to determine the specificity of the probes to the *cpeA* gene, dot blots were performed against DNA from 12 different bacterial species. Hybridization of the DNA probes 1 through 4 occurred only with 8239, 86-11, and 8-6M DNA.

The dot blots probed with probe 1 and probe 4 are shown in Figure 3-4. There was no hybridization against the control organisms *B. subtilis, L. monocytogenes, V. cholera, C. jejuni, E. coli, E. faecalis, S. enteritidis, S. sonnei, Y. enterocolitica,* and *K. oxytoca* indicating that the *cpeA* gene is unique to *C. perfringens*. Interestingly, 8-6M gave a positive result with these probes indicating the presence of nucleic acid sequences unique to the *cpeA* gene. Kokai-Kun *et al.* (1994) found digoxigenin-labeled *cpeA* gene probes and Western immunoblot assays to be 100% specific and sensitive for

Table 3-5. Comparison of *C. perfringens* type A strains 8239, 3624, 86-11, 8-6S, and 8-6M.

	Assay		
	ELISA	Western Immunoblot	Vero cell cytotoxicity
		Controls	
8239	++	+	+
3624	+	-	-
		8-6 Strains	
Group 1			
86-11	++	+	+
8-6S	++	+	+
Group 2			
8-6M	+	-	-

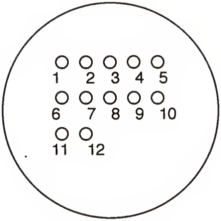
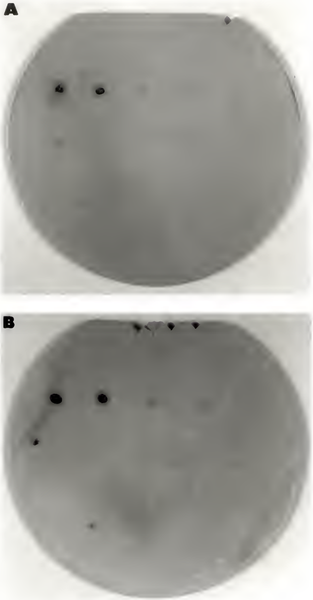


Figure 3-4. Hybridization of probes 1 and 4 to DNA dot blots. The DNA was dot blotted onto the membrane in the pattern shown above. 1. *C. perfringens* type A 8239; 2:*C. perfringens* type A 8-6M; 3: *V. cholerae*; 4:*C. jejuni*; 5: *E. faecalis*; 6: *E. coli;* 7: *E. faecalis*; 8: *K. oxytoca*; 9: *S. sonnei*; 10: *B. subtilis*; 11: *Y. enterocolitica*,12: *L. monocytogenes*. (A) The hybridization of probe 1 to control DNA dot blots. (B) The hybridization of probe 4 to control DNA dot blots.

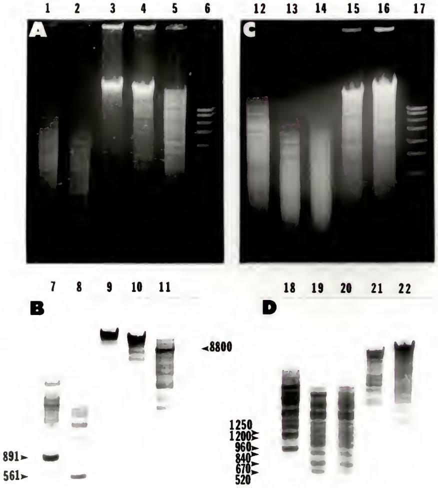

A
B

distinguishing between enterotoxigenic and non-enterotoxigenic *C. perfringens* strains. However, the 8-6M strain gives negative Western analysis and positive gene probe assay results.

Genomic DNA from *C. perfringens* strains 8239, 86-11, and 8-6M were digested with restriction enzymes and analyzed with probes 1 through 4. Genomic DNA from 8239 was resistant to digestion by *Sau*3A and *Bam*HI. Both of these enzymes are inactive if their recognition sequence contains 5-methylcytosine. However, 86-11 and 8-6M genomic DNA was digested by both *Sau*3A and *Bam*HI. All DNA types were digested by the *Sau*3A isoschizomer *Mbo*I, which is sensitive to adenosine methylation, but not cytosine methylation. Therefore, there appears to be a modification system in strain 8239 that is not present in the 8-6 strains. This result confirms similar observations for DNA from strain 8239 made previously (Iwanejko *et al.*, 1989). The modification system has a core recognition sequence of 5'-GATC-3' which results in methylation of the cytosine. This methylation could act as a means of host protection. However, the biological function of cytosine methylation is not well understood (Lewin, 1990).

The DNA restriction enzyme digestion patterns for *C. perfringens* 8239 and 8-6M genomic DNA is shown in Figure 3-5. Southern blot analysis of 8239 and 86-11 DNA revealed *cpeA* probe positive fragments that corresponded to the known *cpeA* gene restriction pattern (Figure 3-1). Hybridization of the *Mbo*I digest of 8239 and 86-11 DNA with probes 1 and 2 revealed a probe positive fragment that corresponded to a 561 bp fragment resulting from *Mbo*I cleavage at positions -215 upstream and 346 (Figures 3-1 and 3-5). Probe 3 showed a 327 bp probe positive fragment resulting from *Mbo*I cleavage at sites 522 and 849 (Figures 3-1 and 3-6). Hybridization with probe 4 showed a positive fragment of 645 bp (Figures 3-1 and 3-6). This corresponds to a fragment

Figure 3-5. Southern blot hybridization of DNA probe 1 to restriction enzyme digests of *C. perfringens* 8239 and 8-6M genomic DNA. (A) *C. perfringens* 8239 restriction enzyme digests. Lane 1: *Hin*dIII digest. Lane 2: *Mbo*I digest. Lane 3: *Sau*3A digest. Lane 4: *Pst*I digest. Lane 5: *Eco*RI digest. Lane 6: MW markers: 8.0, 7.1, 6.0, 4.8, 3.5, 2.7, 1.9, 1.85, 1.5, 1.4 Kb. (B) Lanes 7-11 represent 8239 DNA blotted from lanes 1-5 respectively and hybridized with probe 1. (C) *C. perfringens* 8-6M restriction enzyme digests. Lane 12: *Hin*dIII digest. Lane 13: *Mbo*I digest. Lane 14: *Sau*3A digest. Lane 15: *Pst*I digest. Lane 16: *Eco*RI digest. Lane 17: MW markers: 8.0, 7.1, 6.0, 4.8, 3.5, 2.7, 1.9, 1.85, 1.5, 1.4 Kb. (D) Lanes 18-22 represent 8-6M DNA blotted from lanes 12-16 respectively and hybridized with probe 1.


1 2 3 4 5 6
A
12 13 14 15 16 17
C
7 8 9 10 11
B
◄8800
891►
561►
18 19 20 21 22
D
1250
1200►
960►
840►
670►
520

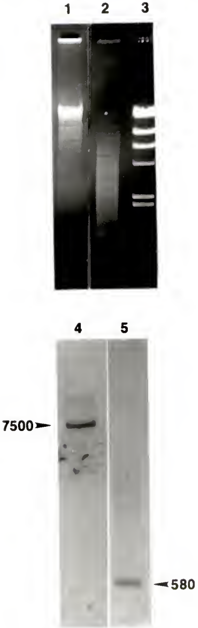


Figure 3-6. Restriction enzyme digests and Southern blots of 8239 DNA. Restriction enzyme digests of 8239 DNA. Lane 1: *Pst*I; lane 2: *Hin*dIII; lane 3: MW markers: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 Kb. Lanes 4 and 5 represent DNA blotted from lanes 1 and 2 respectively and hybridized with probe 4.

resulting from *Mbo*I cleavage at position 849 and a previously unreported *Mbo*I site 534 bases downstream of the *cpeA* gene. Analysis of the *Hin*dIII digest of 8239 and 86-11 DNA showed a probe positive 891 bp fragment which hybridized with probes 1, 2, and 3 (Figure 3-5). This corresponds to a fragment resulting from *Hin*dIII cleavage at sites -182 upstream and 709. Hybridization with probe 4 to the 8239 and 86-11 *Hin*dIII digest resulted in a probe positive fragment of 580 bp (Figure 3-6). This band could correspond to a fragment resulting from *Hin*dIII cleavage at positions 867 and a previously unreported *Hin*dIII site 329 bases downstream of the *cpeA* gene (Figure 3-1). The *Eco*RI digests of 8239 and 86-11 DNA resulted in a single high molecular weight (approximately 8.8 Kb) probe positive fragment with probes 1 through 4 (Figure 3-5). As there is no *Eco*RI site in the *cpeA* gene this result is not unexpected. The *Pst*I digests of 8239 and 86-11 DNA resulted in an approximately 7,500 bp probe positive band with probes 3 and 4. Since there is a *Pst*I site at position 504 within the *cpeA* gene (Figure 3-1), there is most likely a *Pst*I site 7,000 base pairs downstream from this site. Probes 1 and 2 only hybridized with fragments of very high molecular weights (> 10,000 bp) indicating that the first half of the *cpeA* gene is on an even larger *Pst*I fragment.

Hybridization with probes 1 through 4 to restriction enzyme digested 8-6M DNA did not reveal any unique probe positive fragments, as was observed with 8239 and 86-11 DNA (Figure 3-5). It should be noted that there was a high degree of background observed on all Southern blots using probes 1 through 4 due to non-specific hybridization, possibly resulting from the high A+T content of both the *C. perfringens* genome (75%) and the DNA probes (70-80%), and the short length of the probes (30-39 mers). Genomic digests of 8-6M DNA resulted in many probe positive fragments within each digestion (Figure 3-5). Additionally, each type of restriction enzyme digest showed the same banding pattern for each probe. The results from the probed 8-6M restriction digests suggest that all 4 probes based on the *cpeA* nucleotide sequence were binding to regions of 8-6M DNA with only partial homology. Increasing the hybridization stringency between the probes and the 8-6M DNA did not change the results. Probe 1 was constructed using nucleotides 25-54 of the *cpeA* gene which corresponds to amino acids 9-18 of the enterotoxin. The protein sequences of both the CPE-A and CPE 8-6 are believed to be the same through amino acids 9-18 (Martin, 1991). If this is true, then probe 1 would be expected to be homologous to this region of the *cpe86* gene. However, this appears not to be the case.

To resolve the problem of nonspecific probe hybridization to the 86-M DNA, two more probes were synthesized: probes 5 (representing *cpeA* nucleotides 1-126) and 6 (representing *cpeA* nucleotides 49-228). These probes were designed to be much longer than probes 1 through 4 and were used at a hybridization temperature of 47°C instead of 42°C to give a higher specificity for the *cpe* genes.

The combination of longer probes and higher hybridization temperature did increase the specificity of probe binding and also reduced the background. The DNA ladders resulting from *Eco*RI and *Hin*dIII restriction enzyme digestions of 8239 and 86-11 appeared to be similar. The DNA ladders resulting from *Eco*RI and *Hin*dIII restriction enzyme digestions of 86-M genomic DNA were clearly different from that of the 8239 and 86-11 (Figure 3-7). Probe 5 hybridized to a 8.8 Kb fragment of *Eco*RI genomic digests of both 8239 and 86-11 DNA. However, probe 5 did not anneal to any fragment of the *Eco*RI digest of the 8-6M strain (Figure 3-7). Probe 5 also hybridized to a 891 bp fragment of *Hin*dIII genomic digests of 8239 and 86-11 DNA, and did not anneal to any fragment of the 8-6M *Hin*dIII genomic digestion (Figure 3-7).

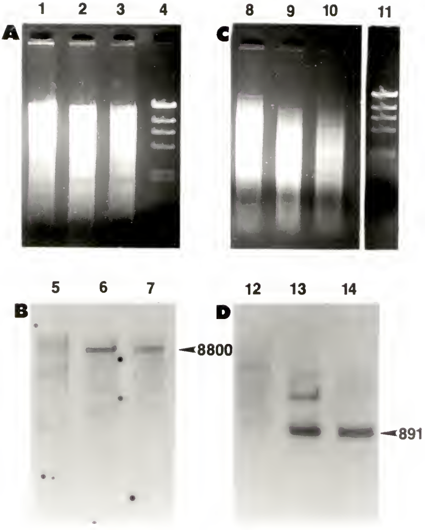


Figure 3-7. Southern blot hybridization of DNA probe 5 to restriction enzyme digests of *C. perfringens* 8239, 86-11, and 8-6M genomic DNA. (A) *Eco*RI restriction enzyme digests. Lane 1: 8-6M DNA; lane 2: 86-11 DNA; lane 3: 8239 DNA; lane 4: MW markers: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, Kb. (B) Lanes 5-7 represent DNA blotted from lanes 1-3 respectively and hybridized with probe 5. (C) *Hin*dIII restriction enzyme digests. Lane 8: 8-6M DNA; lane 9: 86-11 DNA; lane 10: 8239 DNA; lane 11: MW markers: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 Kb. (D) Lanes 12-14 represent DNA blotted from lanes 8-10 respectively and hybridized with probe 5.

Probe 6 did hybridize to fragments of genomic DNA from each strain. Probe 6 hybridized to a 8.8 Kb fragment of *Eco*RI genomic digests of 8239 and 86-11 DNA (Figure 3-8). However, probe 6 hybridized to a 5 Kb fragment of the *Eco*RI genomic digest of 86-M DNA. Probe 6 also hybridized to a 891 bp fragment of *Hin*dIII genomic digests of 8239 and 86-11 DNA, but annealed to a 1,000 bp fragment of the 8-6M *Hin*dIII genomic digestion (Figure 3-8).

The Southern hybridization results suggest the possibility of two discrete groups of 8-6 strains, Group 1 containing the 86-11 and 86-S strains and Group 2 containing the 8-6M strain (Table 3-6). Probe 5, representing *cpeA* nucleotides 1-126, only hybridizes with Group 1 strains, while probe 6, representing *cpeA* nucleotides 49-229, hybridizes with both Groups 1 and 2. Probe 5 differs from probe 6 in that it contains nucleotides 1-49 of the *cpeA* gene, while probe 6 does not. This suggests that the 86-M *cpe86* has different nucleotides than that of the *cpeA* gene or than that of the 86-11 and 86-S *cpe86* gene in positions 1-49. The 86-M *cpe86* gene could have a similar or identical nucleotide sequence to that of the 8239 and 86-11 *cpe* genes from nucleotides 50-229 since probe 6 anneals to all of these strains. The 86-11 strain genomic DNA hybridizes with both probes and the probe positive fragments are the same size as in the 8239 digestions, suggesting the 86-11 *cpe86* is similar to that of the *cpeA*.

The Southern hybridization results also suggest that the Group 2 8-6M carries the *cpe86* gene in a different position on the chromosome than Group 1 strains and the *cpeA* of strain 8239 because probe 6 anneals to different size fragments than that of strains 8239 and Group 1 strains (Table 3-6). To further characterize the position of the *cpe* genes on the chromosome, *Nru*I genomic digestions were performed. It has been observed that when the *cpeA* gene is carried on the chromosome, it occurs on a 5 Kb *Nru*I fragment (Cornillot *et al.*,

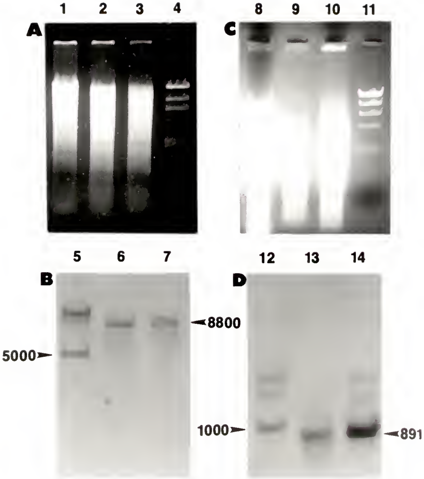


Figure 3-8. Southern blot hybridization of DNA probe 6 to restriction enzyme digests of *C. perfringens* 8239, 86-11, and 8-6M genomic DNA. (A) *Eco*RI restriction enzyme digests. Lane 1: 8-6M DNA; lane 2: 86-11 DNA; lane 3: 8239 DNA; lane 4: MW markers: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, Kb. (B) Lanes 5-7 represent DNA blotted from lanes 1-3 respectively and hybridized with probe 6. (C) *Hin*dIII restriction enzyme digests. Lane 8: 8-6M DNA; lane 9: 86-11 DNA; lane 10: 8239 DNA; lane 11: MW markers: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, Kb. (D) Lanes 12-14 represent DNA blotted from lanes 8-10 respectively and hybridized with probe 6.

Table 3-6. Sizes of probe positive fragments.

Sizes of Probe Positive Fragments

	Genomic DNA Type	
	8239/ 86-11/ 86-S	8-6M
Probe 1		
*Eco*RI	8,800 bp	no one unique fragment
*Hin*dIII	891 bp	no one unique fragment
Probe 4		
*Eco*RI	8,800 bp	no one unique fragment
*Hin*dIII	580 bp	no one unique fragment
Probe 5		
*Eco*RI	8,800 bp	no hybridization
*Hin*dIII	891 bp	no hybridization
*Nru*I	5,000 bp	no hybridization
Probe 6		
*Eco*RI	8,800 bp	5,000 bp
*Hin*dIII	891 bp	1,000 bp
*Nru*I	5,000 bp	hybridization to undigested genomic DNA

1995). Probe 6 did anneal to a 5 Kb fragment of *Nru*I genomic digests in strains 8239 and Group 1 strains. However, probe 6 only hybridized to undigested Group 2 (86-M) DNA (Figure 3-9). Therefore, it appears that Group 1 types and 8239 have similar genome structures while Group 2 strains are distinctly different from either Group 1 or 8239 strains.

The *cpeA* gene is associated with mobile genetic elements (Brynestad *et al.*, 1994) and has been found to be carried on a plasmid (Cornillot *et al.*, 1995). To determine if there was a gene resembling the *cpeA* gene on a plasmid in any of the strains used in this study, plasmid DNA was isolated and subjected to Southern hybridization with probe 6 . Although not shown, the *cpeA* or *cpe86* genes are not carried on a plasmid in strains 8239, 86-11, 8-6S, or 8-6M. There were no detectable probe positive plasmids detected in any of these strains. This result was expected since only strains of non-human origin have been found to carry the *cpeA* gene on a plasmid (Cornillot *et al.*, 1995).

Four sets of PCR primers were synthesized in order to probe the genomes of strains 86-11, 8-6S, and 8-6M for nucleotide sequences that resembled those of the *cpeA* gene. The primers were designed from the published nucleotide sequence of the *cpeA* gene and its promoter region (Czeczulin *et al.*, 1993; Melville, 1994). The primers were designed to different regions of the *cpeA* gene. Since the *cpe86* gene is thought to resemble the *cpeA* gene, it was reasoned that at least one of the primer sets should amplify a region of the *cpe86* that is similar to the *cpeA* gene. The results from the PCR amplification using the 4 primer pairs (Table 3-1 and 3-2) and genomic DNA from 8239, 86-11, 8-6S, and 8-6M are shown in Figure 3-10. Strains 8239, 86-11, and 8-6S did amplify the predicted fragments from all primer pairs while the 8-6M amplified none of the fragments. Since 86-11 and 8-6S amplified all four fragments specific for the *cpeA* gene, these Group 1 strains most likely contain a

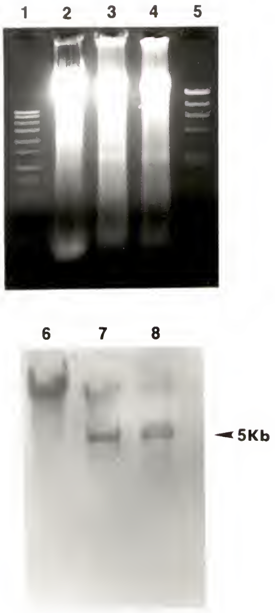
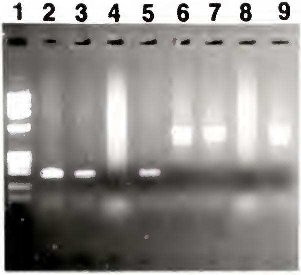
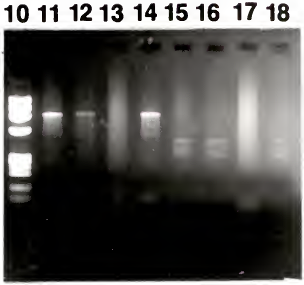


Figure 3-9. Hybridization of probe 6 to *Nru*I digested genomic DNA from 8239, 86-11, and 8-6M. Lane 1: DNA molecular weight markers (Boehringer Mannheim) 8.0, 7.1, 6.0, 4.8, 3.5, 2.7, 1.9, 1.85, 1.5, 1.4, 1.15, 1.0, 0.68, 0.49, 0.37 Kb. Lanes 2-4: *Nru*I digested DNA. Lane 2: 8-6M DNA; lane 3: 86-11 DNA; lane 4: 8239 DNA. Lane 5: MW markers (Promega Corp.): 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, Kb. Lanes 6-8 represent DNA Southern blotted from lanes 2-4 respectively and hybridized with probe 6.

Figure 3-10. PCR amplified fragments from 8239, 86-11, 8-6M, and 8-6S genomic DNA. Lane 1: ϕX 174 DNA/*Hae*III molecular weight marker (Promega Corp.): 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp. Lane 2: primer set A, 8239 DNA; lane 3: primer set A, 86-11 DNA; lane 4: primer set A, 8-6M DNA; lane 5: primer set A, 8-6S DNA; lane 6: primer set B, 8239 DNA; lane 7: primer set B, 86-11 DNA; lane 8: primer set B, 8-6M DNA; lane 9: primer set B, 8-6S DNA; lane 10: ϕX 174 DNA/*Hae*III molecular weight marker; lane 11: primer set C, 8239 DNA; lane 12: primer set C, 86-11 DNA; lane 13: primer set C, 8-6M DNA; lane 14: primer set C, 8-6S DNA; lane 15: primer set D, 8239 DNA; lane 16: primer set D, 86-11 DNA; lane 17: primer set D, 8-6M DNA; lane 18: primer set D, 8-6S DNA.


1 2 3 4 5 6 7 8 9


10 11 12 13 14 15 16 17 18

gene very similar to or exactly the same as the *cpeA*. Strain 8-6M most likely does not carry a gene similar to the *cpeA* gene since no fragments were amplified using the *cpeA* specific primers.

Therefore, there appears to be 2 Groups of 8-6 strains. The Group 1 strains (86-11 and 8-6S) carry a *cpe86* gene that occurs on the chromosome in a similar position to that of the *cpeA* gene in strain 8239. The nucleotide sequence of the Group 1 *cpe86* gene is most likely similar to that of the 8239 *cpeA* gene because probes 5 and 6 react similarly with both the Group 1 DNA and 8239 DNA. The Group 2 (86-M) strain could carry a *cpe86* gene that occurs in a different chromosomal position than that of the *cpeA* in strain 8239 and the *cpe86* in Group 1 strains. If present, the nucleotide sequence of the 8-6M *cpe86* is most likely different than that of the *cpeA* and the *cpe86* of Group 1 strains because probe 6 does specifically anneal to digested 8-6M DNA, but probe 5 does not.

The discovery of two different types of 8-6 strains was unexpected, since the original work by Duncan *et al.* (1972) indicated only a single type. It is possible that a variant has arisen; however, if so its origin is unclear. There are a number of results concerning the 8-6M strain that are perplexing. Strain 8-6M is apparently enterotoxin positive based on ELISA and DNA colony blot studies performed here and in previous work by Martin (1991). Strain 8-6M also reacts with one of several DNA probes specific for the *cpeA* gene, although the hybridization patterns are not completely discrete. Despite these apparent positive results, Western immunoblots, Vero cell cytotoxicity, and PCR analysis of 8-6M do not fully support the existence of an enterotoxin gene in this strain.

The differences between 8-6 strains Group 1 and Group 2 and between all 8-6 strains and 8239 need to be further characterized at the gene level. The presumptive *cpe86* genes from 8-6 strains Group 1 and Group 2 will be cloned and characterized to determine the relationship between these Groups and between 8-6 strains and 8239.

CHAPTER 4
CLONING AND SEQUENCING OF THE ENTEROTOXIN GENES FROM *CLOSTRIDIUM PERFRINGENS* TYPE A 8-6

Introduction

Enterotoxigenic *C. perfringens* type A 8-6 strains can be divided into two groups based on ELISA, Western immunoblot, Vero cell cytotoxicity, PCR analysis, and DNA probe analysis. Group 1 strains of 8-6 resemble the well characterized strain 8239 in all of the above assays. The *cpeA* gene was cloned using a probe positive 5.7 Kb *Xba*I fragment from strain 8239 (Czeczulin *et al.*, 1993). Group 1 strains also revealed a probe positive 5.7 Kb *Xba*I fragment in Southern analysis. Therefore, the Czeczulin *et al.* (1993) cloning methodology was used to clone the *cpe86* gene from Group 1 strain 86-11. Group 2 strains did not resemble the well characterized 8239 strain in the above assays and do not contain a probe positive 5.7 Kb *Xba*I fragment in Southern analysis. However, the *Eco*RI genomic digest of 8-6M DNA resulted in a 4.4 Kb probe 6 positive fragment in Southern analysis. The cloning, sequencing, and characterization of the presumptive enterotoxin genes from 8-6 strains Group 1 and Group 2 are described in this chapter.

Materials and Methods

Strains and Growth Media

Genomic DNA from *C. perfringens* type A 8-6 strains 86-11, 8-6S, and 86-M was prepared as described in the Preparation of *C. perfringens* Genomic

DNA section. *E. coli* DH5α was maintained on Luria Broth (LB) agar (Luria and Delbruck, 1943). *E. coli* transformants carrying the pUC18 plasmid (Boehringer Mannheim Biochemicals) were grown on LB agar supplemented with 50 μg/ml of ampicillin (LA agar). LA broth was the same medium without agar.

Construction of Genomic Library

To isolate an approximately 5.7 Kb probe 5 positive fragment from *Xba*I digested 86-11 DNA, the *Xba*I digested DNA was subjected to electrophoresis using a SeaPlaque GTG (FMC Bioproducts, Rockland ME) preparative agarose gel. The DNA corresponding to 4.4 Kb to 6.6 Kb was cut out of the gel and purified using Agarase (Boehringer Mannheim Biochemicals) according to manufactures directions. An approximately 4.4 Kb probe 6 positive fragment from *Eco*RI digested 8-6M DNA was isolated in the same way.

Approximately 5 μg of the plasmid cloning vector, pUC18, was converted to a linear form by *Xba*I or *Eco*RI and 5' phosphate groups were removed from the DNA molecules with calf intestinal alkaline phosphatase (Promega Corp.) according to manufacturers directions. The pUC18 in a linear form, with 5' phosphate groups removed, was then extracted with phenol/chloroform/ isoamyl alcohol, precipitated with 2.5 volumes of 95% ethanol, and resuspended in 30 μl of TE buffer.

The purified fragments of *C. perfringens* DNA were ligated into their respective restriction sites in pUC18 by T4 DNA ligase (Promega Corp.). Ratios of 1:1, 1:2, 1:5, 2:1, and 3:1 of vector DNA to insert DNA were used, with the 1:3 ratio usually resulting in the greatest number of transformants.

E. coli was made competent for transformation by the calcium chloride procedure and transformed with the ligation mixtures as described by Sambrook *et al.*, (1989) with the following modifications. After the competent cells were mixed with plasmid DNA, they were incubated on ice for 30 minutes, then at 37°C for 2 minutes, then at room temperature for 10 minutes. Two milliliters of LB was added to the transformed cells and the samples were incubated for 1 hour at 37°C to allow phenotypic expression. Transformants were selected by plating the samples of the cells on LA medium and incubating at 37°C overnight.

The transformed colonies were screened for the presence of the *cpe86* gene using colony lifts. Colonies were transferred with toothpicks to two LA plates with numbered grids and incubated overnight at 37°C. A nitrocellulose filter was placed on one of the plates and the colonies were lifted off of the plates. The membrane was placed colony side up on Whatman #3 filter paper saturated with 0.5 N NaOH, 1.5 M NaCl for 15 minutes to lyse the cells. The membrane was transferred to filter paper soaked with 1.0 M Tris-HCl, 1.5 M NaCl, pH 8.0 for 15 minutes. The filter was then treated with UV light to effect crosslinking of the DNA to the filter and the colony debris was gently wiped away with a water soaked paper towel. The prehybridization, hybridization, and detection were completed as described previously. Probes 5 (representing *cpeA* nucleotides 1-126) or 6 (representing *cpeA* nucleotides 49-228) were used for hybridization at 47°C. Probe positive colonies were picked from the second LA plate and grown up in 50 ml of LA broth overnight at 37°C. The plasmids were isolated for restriction enzyme analysis and subcloning using Promega's Magic Mini Preps according to the manufacturers directions.

Subcloning and Sequence Analysis

Plasmids were mapped and subcloned to isolate the smallest region of DNA that resulted in a probe positive reaction. These subcloned plasmids along with plasmids generated from cloning PCR fragments were sequenced in the forward and reverse direction by Mr. Jack Shelton using a LI-COR model 4000L DNA sequencer (Lincoln, NE) operated with the Thermocycle Sequencing kit (Epicenter Technologies, Madison, WI). Sequences were analyzed using the Genetics Computer Group sequence analysis software (Devereux *et al.*, 1984) and the National Center for Biotechnology Information data base BLAST network server (National Library of Medicine via the Internet). Sequence alignments were identified using the Lalign network server (Southern France Human Genome Projects Computing Resource Center via the Internet).

Cloning of PCR Fragments

PCR fragments were purified from agarose gels using Qiaex Gel Extraction kit according to manufacturers directions. These fragments were then cloned using the TA Cloning System (Invitrogen Corp., San Diego, CA) according to manufacturers directions. This kit supplies a vector that provides a single 3' thymine overhang for the easy insertion of PCR fragments that have a single 3' adenine added to all duplex molecules by the non-template dependent activity of the *Taq* polymerase.

Analysis of the Expression of *cpe86* From Recombinant Plasmids

To analyze the expression of the *cpe86* from recombinant plasmids the clone was inoculated into 1 L of LA broth and incubated at 37°C with aeration overnight. The cells were collected by centrifugation, resuspended in 5 ml of lysis buffer consisting of 10 mM EDTA, 25 mM Tris/HCl, 5% glycerol, pH 8.0, and lysed with a Biosonik III Sonicator (Bronwill Scientific, Rochester, NY). The cellular debris was removed by centrifugation and the proteins were isolated by addition of 80% ammonium sulfate, dialysis, and concentration as previously described. The concentrated cellular lysates were subjected to Vero cell cytotoxicity and Western analysis as previously described. *E. coli* DH5α and *E. coli* DH5α transformed with the pUC18 plasmid without an insert were used as controls.

Results and Discussion

The 86-11 *cpe86* gene was cloned using a methodology similar to that used to clone the *cpeA* gene (Czeczulin *et al.*, 1993). Strain 86-11 genomic DNA digested with *Xba*I produced a positive band of 5.7 Kb when Southern blotted and detected with probe 5. Therefore, 86-11 genomic DNA was digested with *Xba*I, subjected to agarose gel electrophoresis, and DNA bands corresponding to 4.4 to 6.6 Kb were cut out of the gel and purified. This DNA was then cloned into the *E. coli* vector pUC18. Recombinant plasmids were then transformed into *E. coli* DH5α and the transformants were screened with probe 5. A probe positive clone was obtained that carried a 5.7 Kb *C. perfringens* DNA insert. The plasmid was isolated and named pLMW1.

An attempt to subclone the probe positive fragment while keeping the whole *cpe86* gene intact was unsuccessful. Restriction enzymes could not be found that would significantly reduce the size of the insert without cutting within the gene. The difficulty in finding a useful restriction enzyme for subcloning may have been due to the high A+T content of the genomic DNA from *C. perfringens* which can be as high as 80%. The *cpeA* gene was also cloned on a 5.7 Kb fragment that was not able to be subcloned (personal communication, Dr. B. McClane). In order to reduce the insert size for sequencing, *Pst*I and *Bgl*II restriction enzymes, which cut within the *cpe86* gene, were used. These enzymes each cut at positions 803 and 646 respectively, within the *cpeA* gene. Plasmid pLMW2 was generated by cutting pLMW1 with *Bgl*II within the *cpe86* gene and at *Bam*HI within the multiple cloning site of pUC18 thereby eliminating intervening fragment. The insert in plasmid pLMW2 was sequenced in the counter clockwise direction. Plasmid pLMW3 was generated by cutting pLMW1 with *Pst*I which cuts within the *cpe86* gene and within the multiple cloning site of pUC18. The intervening fragment was eliminated from the plasmid upon religation. The insert in plasmid pLMW3 was sequenced in the clockwise direction. Plasmid pLMW4 was generated by cloning the isolated 86-11 PCR fragment representing the promoter region of the *cpe86* gene generated by primer set D (Table 3-1 and 3-2). The insert in plasmid pLMW4 was sequenced in both directions. Plasmid pLMW5 was generated by cloning the isolated 86-11 PCR fragment generated by primer set C (Table 3-1 and 3-2) and the insert was sequenced in both directions. Additionally, primer set C was used to amplify a fragment from 8-6S genomic DNA which was cloned, to yield pLMW8. The insert in plasmid pLMW8 was sequenced in both directions. The location of these inserts in relation to the *cpe86* gene are shown in Figure 4-1.

The sequence of *cpe86* (Figure 4-2) cloned from strain 86-11 and its promoter region were identical to that of the 8239 *cpeA*. Additionally, the partial sequence from the 8-6S *cpe86* was identical to that of the 8239 *cpeA* and the 86-11 *cpe86*. The *cpe86* promoter region does not contain the 45 base pair insert that was reported in some strains by Melville *et al.* (1994). Melville *et al.* (1994) also determined using PCR analysis that strain 8239 and strain NCTC 8798 (the strain the 8-6 strain was derived from) did not contain this insert. The gene for the enterotoxin produced by *C. perfringens* type A 86-11 has the same nucleotide sequence as that of the 8239. This result was unexpected since the CPE 8-6 has different activities than the CPE-A. The difference in biological and biochemical activity is therefore not due to differences in the nucleotide coding sequences between the two toxins.

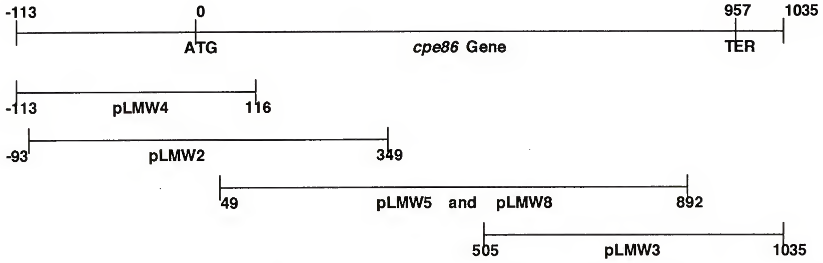


Figure 4-1. The location of *cpe86* subclones in relation to the *cpe86* gene from strain 86-11 and 8-6S.

Figure 4-2. Nucleotide sequence of the *cpe86* gene and the deduced amino acid sequence of the *C. perfringens* type A 86-11 enterotoxin. The putative Shine-Dalgarno ribosome binding site is underlined and indicated by S.D. (Czeczulin *et al.*, 1993). The downstream region containing an inverted repeat that could form a stem-loop structure is indicated by SL.

```
                                          ATAATATAT CACTCTCCTT
        GTTTATAATA TATAATATTA TGTTTAGTGA AATTATGTTA ATATACTACT
        TATTTCTTCT TTTATATTAA TTAACATTTC AACTTGATCT CTTTAACGTA
        TATCTCTTTT ATTACCCAAG CTTTAATTCC TTCAGCATTA ATATCATAAA
        ATGTCCATGT AGAAATATAT TCAAGATTAT TAAAGATATA TATTTTATTT
        AATATTTTGT TAATACTTTA AGGATATGTA TCCAAAATAA AAACTTTTAA
        ATAATATATT ATATAAAAAA AATTAGAAAT AAGGAGATGT TAATTATAAT
                                            SD
1       ATGCTTAGTA ACAATTTAAA TCCAATGGTG TTCGAAAATG CTAAAGAAGT
         M  L  S   N  N  L  N   P  M  V   F  E  N   A  K  E  V
51      ATTTCTTATT TCTGAGGATT TAAAAACACC AATTAATATT ACAAACTCTA
          F  L  I   S  E  D   L  K  T  P   I  N  I   T  N  S N
101     ACTCAAATTT AAGTGATGGA TTATATGTAA TAGATAAAGG AGATGGTTGG
           S  N  L   S  D  G   L  Y  V   I  D  K  G   D  G  W
151     ATATTAGGGG AACCCTCAGT AGTTTCAAGT CAAATTCTTA ATCCTAATGA
         I  L  G   E  P  S  V   V  S  S   Q  I  L   N  P  N  E
201     AACAGGTACC TTTAGCCAAT CATTAACTAA ATCTAAAGAA GTATCTATAA
          T  G  T   F  S  Q   S  L  T  K   S  K  E   V  S  I N
251     ATGTAAATTT TTCAGTTGGA TTTACTTCTG AATTTATACA AGCATCTGTA
           V  N  F   S  V  G   F  T  S   E  F  I  Q   A  S  V
301     GAATATGGAT TTGGAATAAC TATAGGAGAA CAAAATACAA TAGAAAGATC
         E  Y  G   F  G  I  T   I  G  E   Q  N  T   I  E  R  S
351     TGTATCTACA ACTGCTGGTC CAAATGAATA TGTATATTAT AAGGTTTATG
          V  S  T   T  A  G   P  N  E  Y   V  Y  Y   K  V  Y A
401     CAACTTATAG AAAGTATCAA GCTATTAGAA TTTCTCATGG TAATATCTCT
           T  Y  R   K  Y  Q   A  I  R   I  S  H  G   N  I  S
451     GATGATGGAT CAATTTATAA ATTAACAGGA ATATGGCTTA GTAAAACATC
         D  D  G   S  I  Y  K   L  T  G   I  W  L   S  K  T  S
501     TGCAGATAGC TTAGGAAATA TTGATCAAGG TTCATTAATT GAAACTGGTG
          A  D  S   L  G  N   I  D  Q  G   S  L  I   E  T  G E
551     AAAGATGTGT TTTAACAGTT CCATCTACAG ATATAGAAAA AGAAATCCTT
           R  C  V   L  T  V   P  S  T   D  I  E  K   E  I  L
601     GATTTAGCTG CTGCTACAGA AAGATTAAAT TTAACTGATG CATTAAACTC
         D  L  A   A  A  T  E   R  L  N   L  T  D   A  L  N  S
651     AAATCCAGCT GGTAATTTAT ATGATTGGCG TTCTTCTAAC TCATACCCTT
          N  P  A   G  N  L   Y  D  W  R   S  S  N   S  Y  P W
701     GGACTCAAAA GCTTAATTTA CACTTAACAA TTACAGCTAC TGGACAAAAA
           T  Q  K   L  N  L   H  L  T   I  T  A  T   G  Q  K
751     TATAGAATCT TAGCTAGCAA AATTGTTGAT TTTAATATTT ATTCAAATAA
         Y  R  I   L  A  S  K   I  V  D   F  N  I   Y  S  N  N
801     TTTTAATAAT CTAGTGAAAT TAGAACAGTC CTTAGGTGAT GGAGTAAAAG
          F  N  N   L  V  K   L  E  Q  S   L  G  D   G  V  K D
851     ATCATTATGT TGATATAAGC TTAGATGCTG GACAATATGT TCTTGTAATG
           H  Y  V   D  I  S   L  D  A   G  Q  Y  V   L  V  M
901     AAAGCTAATT CATCATATAG TGGAAATTAC CCTTATTCAA TATTATTTCA
         K  A  N   S  S  Y  S   G  N  Y   P  Y  S   I  L  F  Q
951     AAAATTTTAATATTTTAAAATAATATAATCAAATTAATTTACAAAAGACAGTAT
          K  F  *                                         SL
        GTAATATTAAATTATTACATACTGTCTAATT
                          SL
```

The difference in biological and biochemical activity between CPE-A and CPE 8-6 could be attributed to differences in post-translational modifications between the two enterotoxins. Interestingly, CPE-A has been shown to activated threefold when treated with trypsin (Richardson and Granum, 1983) or chymotrypsin (Granum and Richardson, 1991) to a biological activity similar to that of the CPE 8-6. However, trypsinization of the CPE 8-6 results in no increase in activity (Lindsay *et al.*, 1985). The *C. perfringens* type A Group 1 8-6 strains may naturally process the CPE 8-6 protein post-translationally to a mature form thereby making the CPE 8-6 more biologically active than the unmodified CPE-A. Martin (1991) partially sequenced the N-terminus of CPE 8-6 and found the first 42 amino acids were identical to the CPE-A except that it does not contain the first two amino acids (Met and Leu) present in CPE-A.

Only one type A strain *cpeA* gene other than that of the 8239 has been sequenced (Cornillot *et al.*, 1995; Van Damme-Jongsten *et al.*, 1989). Strain F3686 (which is not an 8-6 strain) has eight single nucleotide base differences from strain 8239 in the CPE-A ORF sequence. Seven of these changes are silent at the amino acid level and one of the differences at nucleotide 331, changes the glutamine in the 8239 to glutamic acid in the F3686 *cpeA*. The 8239 *cpeA* sequence has a TAC nucleotide sequence for the codon specifying amino acid 310 of CPE-A, while the F3686 has 2 codons (TCT CAC) at this location. Therefore, the CPE-A of strain F3686 is a 320 amino acid protein while the 8239 CPE-A is a 319 amino acid protein. Additionally, 8239 has an additional T located 93 bases upstream of the first nucleotide of the *cpeA* ORF. There are also differences in the downstream sequence between strains 8239 and F3686. There has been no work completed on the biological activity or biochemical properties of the strain F3686 CPE-A so the effect of these changes on the protein are unknown.

To determine if pLMW1 was expressing the enterotoxin, the lysate from *E. coli* DH5α transformed with pLMW1 was purified, concentrated, and added to Vero cells to determine if a cytotoxic protein was present. The cellular lysate was cytotoxic to Vero cells (Figure 4-3) while the control lysates of *E. coli* DH5α, both with and without the transformed pUC18 vector, were not. Western analysis of the cellular lysate revealed a 35 kDa band that reacted with the CPE 8-6 antisera (Figure 4-4) while the controls did not.

To examine the 8-6 strain Group 2 enterotoxin gene an approximately 4.4 Kb probe 6 positive fragment of 8-6M was cloned into pUC18 and transformed into DH5α. This plasmid was named pLMW6. A single 800 bp probe positive fragment was generated when this plasmid was cut with *Bam*HI. This fragment was subcloned into the *Bam*HI multiple cloning site of pUC18 to generate pLMW7 which was sequenced both in the forward and reverse directions (Figure 4-5). This nucleotide sequence had no open reading frames or sequences with homology to the *cpeA* gene or any other *C. perfringens* gene according to BLAST analysis. Probe 6 revealed 55-58% identity to the cloned fragment in 132 to 193 nucleotide overlaps as determined by the Lalign program. This suggested that probe 6 non-specifically hybridized to digests of 8-6M DNA and that the 8-6 M does not carry a *cpe86* gene. To determine if pLMW6 or pLMW7 were expressing any enterotoxin, the lysates from the clones were purified, concentrated, and added to Vero cells to determine if a cytotoxic protein was present. The cellular lysate from both pLMW6 and pLMW7 were not cytotoxic to Vero cells (Figure 4-3). Western analysis of the cellular lysates did not reveal any 35 kDa band that reacted with the CPE 8-6 antisera (Figure 4-4).

To determine if 8-6M was a 8-6 Group 1 strain that had lost its ability to produce the CPE 8-6, a series of Southern blots of genomic and plasmid DNA

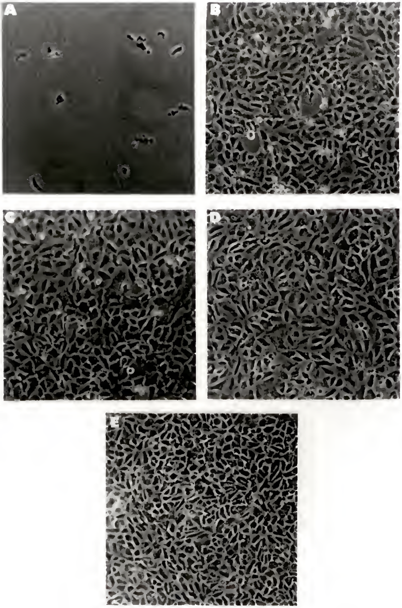


Figure 4-3. The effect of the cellular lysates from pLMW1, pLMW6, pLMW7, DH5α, and DH5α transformed with pUC18 on Vero cells. (A) pLMW1 (B) pLMW6 (C) pLMW7 (D) DH5α (E) DH5α transformed with pUC18.

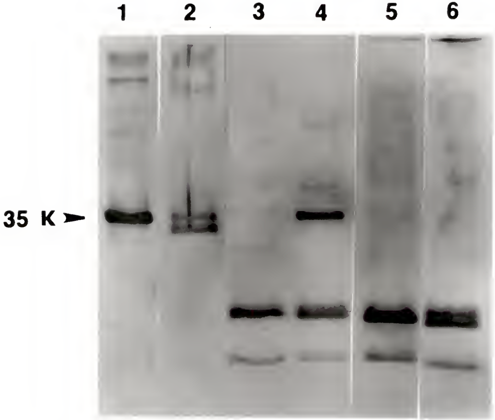


Figure 4-4. Western analysis of cellular lysate from pLMW1, pLMW6, PLMW7, and DH5α transformed with pUC18 detected with a 10^{-3} dilution of antisera to CPE 8-6. The amount of protein in each sample lane is shown in parenthesis. Lane 1: purified CPE-A (0.25 μg); lane 2: purified CPE 8-6 (0.5 μg).; lane 3: DH5α transformed with pUC18 (10 μg) ; lane 4: pLMW1 (14 μg); lane 5: pLMW6 (8 μg); lane 6: pLMW7 (8 μg).

```
1      AAATTTGCCA TTTTTTAATC TCCCCTTTTT TTATTTTTGT TTATATTTTC
51     ACAAAACGCA TTCTAATTAA TATAATTAAA CTGCTTTCTC ATCTGTTTCA
101    TTATCATTGT TTGATTCTGT TTTGCTTCCT AATTCATTAA AGAATATGTT
151    CAGTATAACT GCTGCTAAAC TTCCTGTTGT TATACCACTA TGGAATATTG
201    TTTGCACCCA ATTTGGGAAG TTATGATAAA AGTCAGGATT TGCTAAAGGT
251    ATCATCGCCA ACCCTACACT TATTGCTACA ATTACTCCAT TTTTTGTACC
301    ATTAAACTCA ACTTTGCTTA AAGTTCTTAT TCCACCTACT AAAACCATCC
351    CAAACATGGC GAATCCTACT CCACCAAGAA CCTGTTTAGG TATTGCTGCT
401    ATTATTGATG CAAATTTAGG TATAAATCCT AATACCAATA AGATAATACC
451    TGCATGTATC GCTATGTATC TTGACTTTAT TCCCATCAAC CCAACAAGCC
501    CTGTATTTTG AGCAAATGGA GTTATTGGAA ATGAGTTGAA TATACTAGAT
551    AGAAAAGTTG AAATACCATC TCCTCTTAAT GCTCTTGTTA TATTTGCGCC
601    ATCTATATCT TTATCTACCA TCTCATGTAT TGCCATCATA TTTCCTAATG
651    ATTCAGTCAT TAGTACTAAC ATTATTAAAC CTAAAGATAT TACTGCTGAT
701    GGTTCAAATA CTGGCATACC AAATTTT
```

Figure 4-5. Nucleotide sequence of the probe 6 positive *Bam*HI fragment of 8-6M genomic DNA

were performed. Since the *cpeA* may be on a mobile genetic element, it is not unreasonable to assume the *cpe86* may have been lost or may have moved to a plasmid in the 8-6M strain. However, none of the *C. perfringens* strains used in this study, including 8-6M, carry a plasmid with the *cpe* gene as determined in chapter 3. Therefore, is it probable that strain 8-6M has completely lost the ability to produce CPE 8-6 due to the loss of the *cpe86* gene. This hypothesis can be addressed by analyzing Southern blots done with probe 6 and digested genomic DNA from strains 86-11 and 8-6M. If the 8-6M and 86-11 genomes are the same minus some genes that have moved off the 8-6M chromosome, then strain 86-11 should also show non specific binding of probe 6 to a fragment that is not related to the *cpe86* gene. The 86-11 genome only shows specific binding of probe 6 because a unique probe positive fragment that contained the *cpe86* gene was cloned and sequenced from 86-11 and all of the 86-11 probe positive bands were unique and analogous to the bands in the positive control 8239.

Therefore, strain 8-6M appears not to be a Group 1 8-6 type that has simply lost the *cpe86* gene. It is possible that 8-6M was initially a Group 1 8-6 type that has undergone great modification. In addition to lacking the *cpe86* gene and drastically different DNA ladders than Group 1, it has the unique characteristic of digestion with restriction enzyme *Sau*3A. This characteristic differentiates 8239 from 8-6 types.

Therefore, Group 1 strains of *C. perfringens* carry a *cpe86* gene that has the same nucleotide sequence of that of the 8239 *cpeA* gene. However, the CPE 8-6 protein is biologically and biochemically different from that of the CPE-A. A possible explanation for this observation could be that the CPE 8-6 is post-translationally modified to a different form and folded differently than that of the CPE-A. This possibility will be explored in Chapter 5.

CHAPTER 5
MATRIX-ASSISTED LASER DESORPTION IONIZATION MASS SPECTROMETRY ANALYSIS OF THE CPE-A AND CPE 8-6 PROTEINS

Introduction

The enterotoxin CPE 8-6 produced by the *C. perfringens* type A 8-6 strain differs biologically and biochemically from the enterotoxin CPE-A produced by the 8239 strain (McClane, 1994; Lindsay *et al.*, 1985; Mach and Lindsay, 1994). However, data obtained during this study indicates that the nucleotide sequence of the gene coding for CPE 8-6 is the same as the gene coding for CPE-A. This result suggests that the different properties of the two proteins may result from post-translational modifications.

The sequence of the CPE-A protein has been deduced from the nucleic acid sequence. An N-terminal sequence analysis of CPE 8-6 indicated complete homology between this protein and CPE-A for the first 42 amino acid residues except CPE 8-6 lacked the Met and Leu residues at positions 1 and 2 (Martin, 1991). The nucleotide sequence of the *cpe86* gene obtained during this study indicated that the codons for these 2 amino acids do occur at positions 1 and 2. Therefore, the putative protein sequence for the CPE 8-6 derived from the nucleic acid sequence is the same as for the CPE-A. However, the CPE 8-6 may be altered in some fashion resulting in the loss of these first 2 amino acids.

Little is known of the secondary, tertiary, or quaternary structure of the CPE-A protein. Analysis by UV circular dichroism predicted 80% β sheet and 20% α helix secondary structure (Granum and Harbitz, 1985). Granum and

Stewart (1993) attempted to predict secondary structure by using 9 different structure prediction models and obtained conflicting putative conformations that were consistent in only 2 areas. The N-terminal residues 10-25 were predicted to conform to an α-helix in 8 of the 9 models. Biochemical analysis supports this conformation since this region is available for cleavage by trypsin and chymotrypsin. A putative α-helix conformation is also predicted between residues 195 and 215 in all models. The CPE-A has 43% hydrophobic amino acids resulting in the tendency of the protein to aggregate both *in vivo* and *in vitro*. CPE-A can form crystalline inclusions in the mother cells during sporulation, thereby resulting in some quaternary structure. Interestingly, the only other naturally occurring crystalline inclusion known to occur in bacteria is the insecticidal δ-endotoxin of *Bacillus thuringiensis* (Granum and Stewart, 1993). Aggregation of the CPE-A also occurs *in vitro* even in the presence of SDS. Despite the propensity of CPE-A to aggregate, suitable crystals for X-ray analysis have not been obtained. The three dimensional structure of the CPE-A has been analyzed by UV-differential spectroscopy and by the titration of accessible amino groups, and a probable 2 domain structure was predicted (Granum and Whitaker, 1980; Whitaker and Granum, 1980). The work of McClane (1994) supports the two-domain model. Nothing is known of the secondary, tertiary, or quaternary structure of the CPE 8-6.

To determine if the differences between the two proteins are due to alterations in protein structure, CPE-A and CPE 8-6 were analyzed by matrix-assisted laser desorption (MALD) ionization mass spectrometry (Karas *et al.*, 1985; Beavis and Chait, 1989). This technique allows the mass spectral analysis of minute amounts of protein by desorption and ionization of many protein molecules resulting from illumination of a supporting matrix with UV laser light. The resulting ions acquire a constant kinetic energy after acceleration in the ion source allowing their masses to determined by their flight time to a fixed ion detector (Beavis and Chait, 1989).

Materials and Methods

Digestion of the Proteins

Purified CPE-A was obtained from Dr. Bruce McClane, University of Pittsburgh, and purified CPE 8-6 from Dr. James Lindsay. Protein fragments generated by protease digestion were analyzed using the PROCOMP Version 1.2 program obtained from Dr. P. C. Andrews, University of Michigan Medical School, Ann Arbor, MI. Freeze dried CPE-A and CPE 8-6 were resuspended in water to a concentration of 3.2 and 2.4 mg/ml respectively. Samples were either digested with a protease or analyzed in the undigested form. For digestion, 10 μl of the samples were added to 10 μl of 2 x digestion buffer (0.1 M bicarbonate buffer, pH 8.1). Then either 1.6 μg of sequencing grade endoproteinase GluC (GluC) (Sigma Chemical Co.), which cleaves after each glutamic acid residue, or 2.6 μg of sequencing grade trypsin (Sigma Chemical Co.), which cleaves after each lysine and arginine residue, was added to CPE-A and CPE 8-6 samples and incubated 24 hours at 37°C with gentle shaking. One microliter of sample and 1 μl of 0.1 M matrix, 3,5-dimethoxy-4-hydroxycinnamic acid (SA), were added to and mixed on the target and allowed to dry. The samples were then analyzed by MALD ionization mass spectrometry.

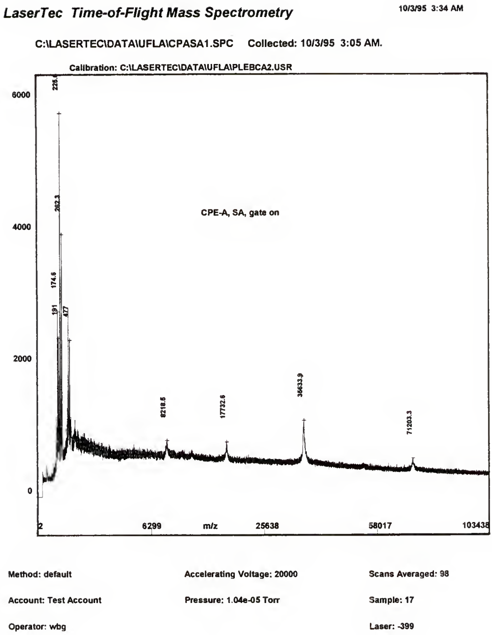
Matrix-Assisted Laser Desorption (MALD) Ionization Mass Spectrometry

A Vestec (Houston, TX) LaserTec matrix-assisted laser desorption (MALD) mass spectrometer was used to obtain the spectra. The spectrometer contains a N_2 laser and a 1.2 meter flight tube. The accelerating voltage was 10 or 20 kv. Approximately 75 spectra were averaged to obtain the displayed spectra.

Results and Discussion

The native molecular weights (MW) of CPE-A and CPE 8-6 were determined by MALD (Figure 5-1 and Figure 5-2). CPE-A has a MW of 35,634 Da which is in good agreement with the MW of 35,317 Da obtained by sequence analysis. The $(M+2H)^{+2}$ and $(M+3H)^{+3}$ ion peaks are present at 17,733 and 8,219 Da respectively. Additionally, a $(2M+H)^{+}$ ion peak was observed a t 71,203 Da. The CPE 8-6 spectra reveals 2 peptides of 35,237 and 32,685 Da. These 2 CPE 8-6 peptides can be visualized as 35,000 and 33,000 Da bands in Western analysis of purified CPE 8-6 (Figure 4-4). Therefore, the 32,685 Da peptide appears to be a fragmented form of CPE 8-6. Additionally, a peak at 4,916 Da was observed. This peak was not observed in the CPE-A spectra and is unexplained.

The mass spectra for trypsin treated native CPE-A is shown in Figure 5-3. Peptide signals were observed at 32,438 and 16,149 Da. The 32,438 Da peptide most likely represents CPE-A protein that has been cleaved after residue Lys 25 which would have a predicted MW of 32,424 Da. This suggests that residue 25 is exposed on the surface of the enterotoxin molecule. The accessibility of residue 25 has been noted by others and cleavage at this residue has been shown to activate the biological activity of the enterotoxin threefold (Rood and Cole, 1991). The 16,149 Da peptide represents about half of the 32,438 Da peptide and could represent any one of the 8 predicted trypsin generated peptides of approximately 16,400 Da. Alternatively, the 16,149 Da peak could represent the $(M+2H)^{+2}$ ion.



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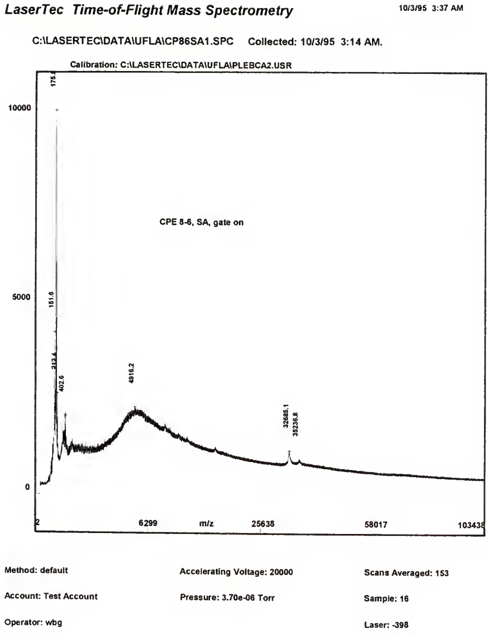
Calibration: C:\LASERTEC\DATA\UFLA\PLEBCA2.USR

Method: default **Accelerating Voltage: 20000** **Scans Averaged: 98**

Account: Test Account **Pressure: 1.04e-05 Torr** **Sample: 17**

Operator: wbg **Laser: -399**

Figure 5-1. MALD ionization mass spectra for undigested, native CPE-A using SA matrix.



LaserTec Time-of-Flight Mass Spectrometry 10/3/95 3:37 AM

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Calibration: C:\LASERTEC\DATA\UFLA\PLEBCA2.USR

CPE 8-6, SA, gate on

Method: default

Account: Test Account

Operator: wbg

Accelerating Voltage: 20000

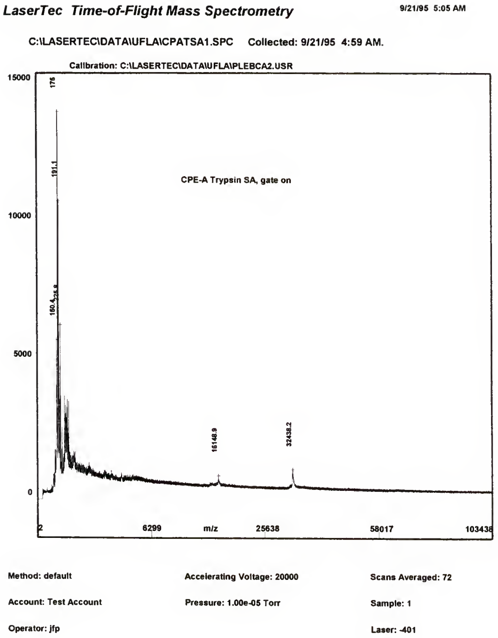
Pressure: 3.70e-06 Torr

Scans Averaged: 153

Sample: 16

Laser: -398

Figure 5-2. MALD ionization mass spectra for undigested, native CPE 8-6 using SA matrix.



LaserTec Time-of-Flight Mass Spectrometry 9/21/95 5:05 AM

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Calibration: C:\LASERTEC\DATA\UFLA\PLEBCA2.USR

Method: default

Account: Test Account

Operator: jfp

Accelerating Voltage: 20000

Pressure: 1.00e-05 Torr

Scans Averaged: 72

Sample: 1

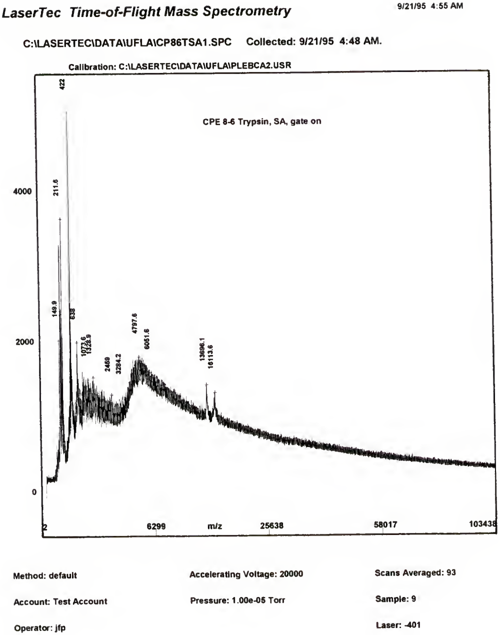
Laser: -401

Figure 5-3. MALD ionization mass spectra for native CPE-A digested with trypsin and using SA matrix.

Interestingly, the undigested CPE 8-6 has peaks at 35,237 and 32,685 Da. The 32,685 Da peptide is similar in size to the to the trypsin digested CPE-A 32,438 Da peptide. Since the biological activity of CPE-A is activated threefold from 112 μg/kg to 38 μg/kg when treated with trypsin, and trypsinization of CPE 8-6 results in no increase in its biological activity of 39 μg/kg, CPE 8-6 may be synthesized as a 319 amino acid protein that is subsequently modified by cleavage of the 25 N-terminal amino acids *in vivo* by a strain specific enzyme to the higher biological activity of the CPE-A that has been treated with trypsin. The rCPE 8-6 synthesized in *E. coli* is present only as a 35 kDa species that migrates on SDS-PAGE with the 35 kDa CPE 8-6 species (Figure 4-4). The absence of the 32 kDa species in rCPE produced in *E. coli* provides further evidence that the *C. perfringens* type A 86-11 strain produces an enzyme that post-translationally processes the CPE 8-6, that is not present in the type A 8239 strain or in *E. coli*. Alternatively, the purified CPE 8-6 may have been modified from the 35 kDa to the 32 kDa species during purification or storage.

The mass spectra for native CPE 8-6 treated with trypsin is different than that of the CPE-A. Peptide signals were observed at 13,696 and at 15,114 Da (Figure 5-4). Since CPE 8-6 already may have a modification that mimics trypsinization it could be possible that after treatment with trypsin there is more digestion of CPE 8-6 than is observed for CPE-A. Alternatively, CPE 8-6 may be folded differently than the CPE-A exposing different trypsin digestion sites than CPE-A.

The mass spectra for native CPE-A treated with GluC is shown in Figure 5-5. Peptide signals were observed at 32,888, 31,863, 16,581, 15,871, 13,559, 4,804, and 2,313 Da. This indicates that several residues are available for cleavage by GluC. The mass spectra for native CPE 8-6 treated with GluC is



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Method: default

Account: Test Account

Operator: jfp

Accelerating Voltage: 20000

Pressure: 1.00e-05 Torr

Scans Averaged: 93

Sample: 9

Laser: -401

Figure 5-4. MALD ionization mass spectra for native CPE 8-6 digested with trypsin and using SA matrix.

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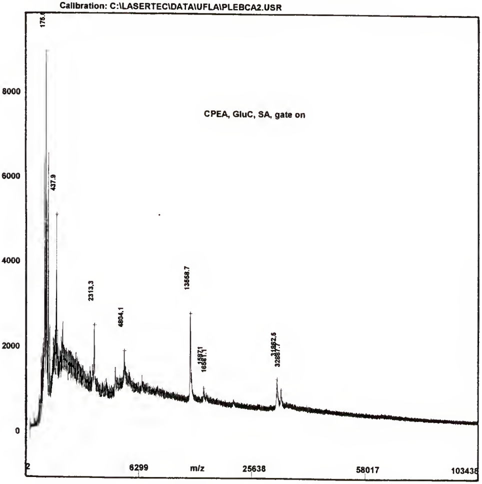


Method: default

Account: Test Account

Operator: jfp

Accelerating Voltage: 20000

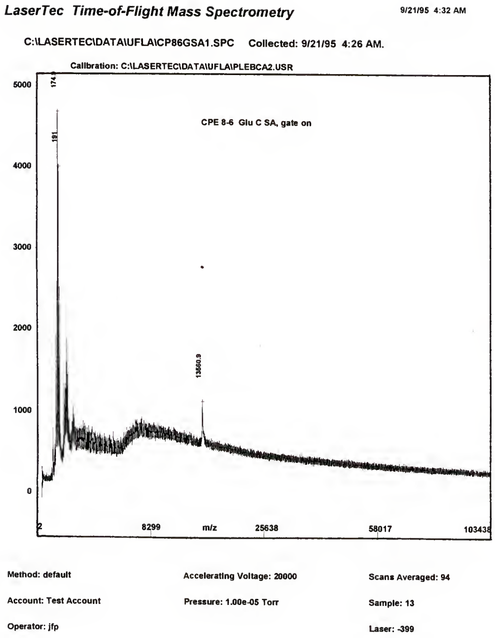
Pressure: 2.33e-06 Torr

Scans Averaged: 66

Sample: 6

Laser: -399

Figure 5-5. MALD ionization mass spectra for native CPE-A digested with GluC and using SA matrix.



LaserTec Time-of-Flight Mass Spectrometry **9/21/95 4:32 AM**

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Calibration: C:\LASERTEC\DATA\UFLA\PLEBCA2.USR

Method: default

Account: Test Account

Operator: jfp

Accelerating Voltage: 20000

Pressure: 1.00e-05 Torr

Scans Averaged: 94

Sample: 13

Laser: -399

Figure 5-6. MALD ionization mass spectra for native CPE 8-6 digested with GluC and using SA matrix.

shown in Figure 5-6. The only peptide signal was observed at 13,561 Da. This is in contrast to the many signals observed for the CPE-A. This could indicate that only a few residues are available for cleavage by GluC. However, smaller peptides may not have been detected.

In summary, these preliminary studies suggest that in the native conformation the CPE-A and CPE 8-6 proteins differ. The differences between CPE-A and CPE 8-6 may be due to different post-translational modifications or to conformational differences as a result of the post-translational modifications. Alternatively, modifications during purification or storage may have resulted in changes to the CPE 8-6. Studies are continuing to resolve these questions.

CHAPTER 6
CONCLUSIONS AND SUMMARY

The goal of the research presented in this dissertation was to compare and contrast the type 8-6 enterotoxin from strain NCTC 8798 to that of the type A enterotoxin from strain NCTC 8239. *C. perfringens* type A and type A 8-6 strains were screened by ELISA, Western immunoblot, Vero cell cytotoxicity, and PCR analysis to determine enterotoxigenic activity. Restriction enzyme profiles and Southern analysis were used to identify any differences. No differences in restriction enzyme profiles were observed between the genomic DNA from the type A strain, 8239, and the type A 8-6 strain, 86-11. The chromosomal locations of the *cpeA* and *cpe86* genes were also determined. In both the type A strain and the type A 8-6 strain the enterotoxin gene was present on a 5 Kb *Nru*I, a 5.7 Kb *Xba*I, and a 8.8 Kb *Eco*RI restriction enzyme fragment.

The *cpe86* gene was cloned and sequenced in order to compare it to the *cpeA* gene. The nucleic acid sequence of the *cpe86* gene and its upstream region (to 319 bases upstream) was homologous to the *cpeA* gene. This result was unexpected since CPE 8-6 exhibits different characteristics than the CPE-A. A possible explanation for this result was that the CPE 8-6 protein may have may be post-translationally modified differently than CPE-A. Post-translational modification with trypsin has been shown to increase the biological activity of the CPE-A; however, trypsin has no effect on the biological activity of CPE 8-6.

Preliminary studies using matrix-assisted laser desorption (MALD) ionization mass spectrometry suggest CPE 8-6 may be synthesized as a 319 amino acid protein and post-translationally modified *in vivo* by an enzyme that cleaves in a manner similar to trypsin. The differences observed in biochemical properties and biological activity between CPE-A and CPE 8-6 may have been due to post-translational modification or conformational differences resulting from the post-translational modification of the CPE 8-6.

Future experiments should focus on characterizing the secondary and tertiary structure of CPE 8-6 and CPE-A proteins using MALD ionization mass spectrometry. Peptides generated from protease treated CPE 8-6 and CPE-A could be isolated and sequenced to provide insight into the structure of both enterotoxins. This information is important since little is known about the secondary or tertiary structure of either enterotoxin. Information on the structure will be important in delineating how these molecules function as enterotoxins and superantigens and how they interact with the host defense system.

REFERENCES

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1989) Current Protocols in Molecular Biology. New York: Wiley-Interscience

Baez LA, Juneja VK (1995) Detection of enterotoxigenic *Clostridium perfringens* in raw beef by polymerase chain reaction. J Food Prot 58:154-159

Bean NH, Griffin PM (1990) Foodborne disease outbreaks in the United States, 1973-1987: Pathogens, vehicles, and trends. J Food Prot 53:804-817

Beavis RC, Chait BT (1989) Cinnamic acid derivatives as matrices for ultraviolet laser desorption mass spectrometry of proteins. Rapid Commun Mass Spectrom 3:432-435

Berry PR, Rodhouse JC, Hughs S, Bartholomew BA, Gilbert RJ (1988) Evaluation of ELISA, RPLA, and Vero cell assays for detecting *Clostridium perfringens* in faecal specimens. J Clin Pathol 41:458-461

Bartholomew BA, Stringer MF, Watson GN, Gilbert RJ (1985) Development and application of an enzyme linked immunosorbent assay for *Clostridium perfringens* type A enterotoxin. J Clin Pathol 38:222-228

Betley MJ, Harris TO (1994) Staphylococcal enterotoxins: Genetic characterization and relationship between structure and emetic activity. Food Microbiol 11:109-121

Boriello SP, Barclay FE, Welch AR, Stringer MF, Watson GN, Williams RKT, Seal DV, Sullens K (1985) Epidemiology of diarrhoea caused by enterotoxigenic *Clostridium perfringens*. J Med Microbiol 20:363-372

Borriello SP, Larson HE, Welch AR, Barclay F, Stringer MF, Bartholomew BA (1984) Enterotoxigenic *Clostridium perfringens*: A possible cause of antibiotic-associated diarrhoea. Lancet 1:305-307

Bowness P, Moss PAH, Tranter H, Bell JI, McMichael AJ (1992) *Clostridium perfringens* enterotoxin is a superantigen reactive with human T cell receptors Vβ6.9 and Vβ22. J Exp Med 176:893-896

Brynestad S, Iwanejko LA, Stewart GSAB, Granum PE (1994) A complex array of Hpr consensus DNA recognition sequences proximal to the enterotoxin gene in *Clostridium perfringens* type A. Microbiol 140:97-104

Bunning VK (1994) Immunopathogenic aspects of foodborne microbial disease. Food Microbiol 11:89-95

Byun MOK, Kaper JB, Ingram LO (1986) Construction of a new vector for the expression of foreign genes in *Zymomonas mobilis*. J Ind Microbiol 1:9-15

Canard B, Saint-Joanis B, Cole ST (1992) Genomic diversity and organization of virulence genes in the pathogenic anaerobe *Clostridium perfringens*. Mol Microbiol 6:1421-1429

Cato EP, George WL, Finegold SM (1986) Genus *Clostridium*. In: Sneath PHA (ed) Bergey's Manual of Systematic Bacteriology Vol. 2. Baltimore, MD: Williams and Wilkins, pp 1141-1200

Centers for Disease Control (1994) *Clostridium perfringens* gastroenteritis associated with corned beef served at St. Patrick's Day meals-Ohio and Virginia, 1993. Morb Mort Week Rep 43(8):137-144

Collie R, Czeczulin J, McClane B (1995) Comparison of expression/regulation of the *Clostridium perfringens* enterotoxin gene (*cpe*) after electroporation into *cpe*-negative isolates of *Clostridium perfringens* type A, B, and C. Presented at the meeting of the American Society for Microbiology First International Conference on the Molecular Genetics and Pathogenesis of the Clostridia, Rio Rico, AZ

Cornillot E, Saint-Joanis B, Daube G, Katayama S, Granum PE, Canard B, Cole ST (1995) The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. Mol Microbiol 15:639-647

Czeczulin JR, Hanna PC, McClane BA (1993) Cloning, nucleotide sequencing, and expression of the *Clostridium perfringens* enterotoxin gene in *Escherichia coli*. Infect Immun 61:3429-3439

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence anlysis programs for the VAX. Nucleic Acids Res 12:387-395

Duncan CL, Strong DH, Sebald M (1972) Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. J Bacteriol 110:378-391

Eisgruber H, Wiedmann M, Stolle A (1995) Use of plasmid profiling as a typing method for epidemiologically related *Clostridium perfringens* isolates from food poisoning cases and outbreaks. Lett Appl Microbiol 20:290-294

Fleischer B (1994) Superantigens. APMIS 102:3-12

Granum PE, Harbitz O (1985) A circular dichroism study of the enterotoxin from *Clostridium perfringens* type A. J Food Biochem 9:137-146

Granum PE, Richardson M (1991) Chymotrypsin treatment increases the activity of *Clostridium perfringens* enterotoxin. Toxicon 29:898-900

Granum PE, Stewart GSAB (1993) Molecular biology of *Clostridium perfringens* enterotoxin. In: Sebald M (ed) Genetics and Molecular Biology of Anaerobic Bacteria. New York: Springer-Verlag, pp 235-247

Granum PE, Whitaker JR (1980) Improved method for purification of enterotoxin from *Clostridium perfringens* type A. Appl Environ Microbiol 39:1120-1122

Granum PE, Whitaker JR, Skjelkvale R (1981) Trypsin activation of enterotoxin from *Clostridium perfringens* type A: Fragmentation and some physiocochemical properties. Biochem Biophys Acta 668:325-332

Hanna PC, McClane BA (1991) A recombinant C-terminal toxin fragment provides evidence that membrane insertion is important for *Clostridium perfringens* enterotoxin cytotoxicity. Mol Microbiol 5:225-230

Hanna PC, Mietzner TA, Schoolnik GK, McClane BA (1991) Localization of the receptor-binding region of *Clostridium perfringens* enterotoxin utilizing cloned toxin fragments and synthetic peptides: The 30 C-terminal amino acids define a functional binding region. J Biol Chem 266:11037-11043.

Hanna PC, Wieckowski EU, Mietzner TA, McClane BA (1992) Mapping of functional regions of *Clostridium perfringens* type A enterotoxin. Infect Immun 60:2110-2114

Hanna PC, Wnek AP, McClane BA (1989) Molecular cloning of the 3' half of the *Clostridium perfringens* enterotoxin gene and demonstration that this region encodes the receptor-binding activity. J Bacteriol 171:6815-6820

Hathaway CL (1990) Toxigenic Clostridia. Clin Microbiol Rev 3:66-98

Hauschild AHW, Thatcher FS (1967) Experimental food poisoning with heat-susceptible *Clostridium perfringens* type A. J Food Sci 32:467-469

Hauschild AH, Niilo L, Doward WJ (1971) The role of enterotoxin in *Clostridium perfringens* type A enteritis. Can J Microbiol 17:987-991

Heredia NL, Labbe RG, Rodriguez MA, Garcia-Alavarado JS (1991) Growth, sporulation and enterotoxin production by *Clostridium perfringens* type A in the presence of human bile salts. FEMS Microbiol Lett 84:15-22

Hobbs BC, Smith ME, Oakley CL, Warrack GH, Cruickshank JC (1953) *Clostridium welchii* food poisoning. J Hyg 51:75-101

Hulkower KI, Wnek AP, McClane BA (1989) Evidence that alterations in small molecule permeability are involved in the *Clostridium perfringens* type A enterotoxin-induced inhibition of macromolecular synthesis in Vero cells. J Cell Physiol 140:498-504

Iwanejko LA, Routledge MN, Stewart GSAB (1989) Cloning in *Escherichia coli* of the enterotoxin gene from *Clostridium perfringens* type A. J Gen Microbiol 135:903-909

Jackson SG, Yip-Chuck DA, Clark JB, Brodsky MH (1986) Diagnostic importance of *Clostridium perfringens* enterotoxin analysis in recurring enteritis among elderly, chronic care psychiatric patients. J Clin Microbiol 23:748-751

Johnson EA (1990) *Clostridium perfringens* food poisoning. In: Cliver DO (ed.) Foodborne Diseases. New York: Academic Press, Inc., pp 229-240

Kallio PT, Fagelson JE, Hoch JA, Strauch MA (1991) The transition state regulator Hpr of *Bacillus subtilis* is a DNA-binding protein. J Biol Chem 266:13411-13417

Karas M, Bachmann D, Hillenkamp F (1985) Influence of the wavelength in high-irradiance ultraviolet lasar desorption mass spectrometry of organic molecules. Anal Chem 57:2935-2939

Katsuno S, Kondo M (1973) Regulation of staphylocccal enterotoxin B synthesis and its relation to other extracellular proteins. Jap J Med Sci Biol 26:26-29

Kokai-Kun JF, Songer JG, Czeczulin JR, Chen F, McClane BA (1994) Comparison of western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens*. J Clin Microbiol 32:2533-2539

Kotzin BL, Leung DYM, Kappler J, Marrack P (1993) Superantigens and their potential role in human disease. Adv Immunol 54:99-166

Labbe RG (1980) Relationship between sporulation and enterotoxin production in *Clostridium perfringens* type A. Food Technol 34(4):88-90

Labbe RG (1989) *Clostridium perfringens*. In: Doyle MP (ed) Foodborne Bacterial Pathogens. New York: Marcel Dekker, Inc.

Labbe RG Duncan CL (1977) Evidence for stable messenger ribonucleic acid during sporulation and enterotoxin synthesis by*Clostridium perfringens* type A. J Bacteriol 129:843-849

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680-685

Larson HE, Borriello SP (1988) Infectious diarrhea due to *Clostridium perfringens*. J Infect Dis 157:390-391

Lewin B (1990) Genes IV. New York: Oxford University Press

Lindsay JA (1988a) Characterization of bacterial spores from high-temperature growth transformants of *Bacillus subtilis*. Curr Microbiol 16:265-269

Lindsay JA (1988b) The effect of a *Clostridium perfringens* 8-6 enterotoxin on viability and macromolecular synthesis in Vero cells. Biochem Biophys Res Comm 151:1371-1377

Lindsay JA, Dennison JD (1986a) Histopathological effect of *Clostridium perfringens* 8-6 enterotoxin on rabbit intestine. Curr Microbiol 13:61-66

Lindsay JA, Dennison JD (1986b) A scanning electron microscope study of the effect of an enterotoxin from *Clostridium perfringens* 8-6 on mice of different ages. J Gen Microbiol 132:2893-2898

Lindsay JA, Johnson HM, Wallace FM, Soos JM (1994) Can superantigens trigger sudden infant death? Med Hypoth 43:81-85

Lindsay JA, Mach AS, Wilkinson MA, Martin LM, Wallace FM, Keller AM, Wojciechowski LM (1993) *Clostridium perfringens* type A cytotoxic-enterotoxin(s) as triggers for death in the sudden infant death syndrome: Development of a toxico-infection hypothesis. Curr Microbiol 27:51-59

Lindsay JA, Sleigh RW, Ghitgas C, Davenport JB (1985) Purification and properties of an enterotoxin from a coatless spore mutant of *Clostridium perfringens* type A. Eur J Biochem 149:287-293

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275

Lund BM (1990) Foodborne disease due to *Bacillus* and *Clostridium* species. Lancet 336:982-986

Luria SE, Delbruck M (1943) Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28:491-511

Mach AS, Lindsay JA (1994) Activation of *Clostridium perfringens* cytotoxic enterotoxin(s) *in vivo* and *in vitro*: Role in triggers for sudden infant death. Curr Microbiol 28:261-267

Mahony DE, Ahmed R, Jackson SG (1992) Multiple typing techniques applied to a *Clostridium perfringens* food poisoning outbreak. J Appl Bacteriol 72:309-314

Martin LM (1991) Cloning of the *Clostridium perfringens* type A (8-6) enterotoxin gene into *Escherichia coli* Chi 1776. MS Thesis, University of Florida, Gainesville

McClane BA (1984) Osmotic stabilizers differentially inhibit permeability alterations induced in Vero cells by *Clostridium perfringens* enterotoxin. Biochim Biophys Acta 777:99-106

McClane BA (1992) *Clostridium perfringens* enterotoxin: structure, action and detection. J Food Safety 12:237-252

McClane BA (1994) *Clostridium perfringens* enterotoxin acts by producing small molecule permeability alterations in plasma membranes. Toxicol 87:43-67

McClane B (1995) ASM First International Conference on the Molecular Genetics and Pathogenesis of the Clostridia: Progress in understanding clostridial toxins and their genes could reduce the damage they cause. ASM News 61:465-468

McClane BA, McDonel JL (1980) Characterization of membrane permeability alterations induced in Vero cells by *Clostridium perfringens* enterotoxin. Biochim Biophys Acta 600:974-985

McClane BA, Wnek AP (1990) Studies of *Clostridium perfringens* enterotoxin action at different temperatures demonstrate a correlation between complex formation and cytotoxicity. Infect Immun 58:3109-3115.

McClane BA, Wnek AP, Hulkower KI, Hanna PC (1988) Divalent cation involvement in the action of *Clostridium perfringens* type A enterotoxin: Early events in enterotoxin action are divalent cation independent. J Biol Chem 263:2423:2435

McDonel JL, (1980a) Binding of *Clostridium perfringens* [^{125}I] enterotoxin to rabbit intestinal cells. Biochem 19:4801-4807

McDonel JL (1980b) *Clostridium perfringens* toxins (type A, B, C, D, E). Pharmac Ther 10:617-655

McDonel JL, Chang LW, Pounds JG, Duncan CL (1978) The effects of *Clostridium perfringens* on rat and rabbit ileum: an electron microscopy study. Lab Invest 39:210-218

Mekalanos JJ (1992) Environmental signals controlling expression of virulence determinants in bacteria. J Bacteriol 174:1-7

Melville SB, Labbe R, Sonenshein AL (1994) Expression from the *Clostridium perfringens cpe* promoter in *C. perfringens* and *Bacillus subtilis*. Infect Immun 62:5550-5558

Murrell WG (1989) *Clostridium perfringens*. In: Buckle K (ed) Foodborne Microorganisms of Public Health Significance. Pymble, Australia: Australian Institute of Food Science and Technology, Ltd., pp 209-232

Niilo L (1975) Measurement of biological activities of purified and crude enterotoxin of *Clostridium perfringens*. Infect Immun 12:440-442

Richardson M, Granum PE (1983) Sequence of the amino-terminal part of enterotoxin from *Clostridium perfringens* type A: Identification of points of trypsin activation. Infect Immun 40:943-949

Richardson M, Granum PE (1985) The amino acid sequence of the enterotoxin of *Clostridium perfringens* type A. FEBS Lett 182:479-484

Roberts I, Holmes WM, Hylemon PB (1986) Modified plasmid isolation method for *Clostridium perfringens* and *Clostridium absonum*. Appl Env Microbiol 52:197-199

Rood JI, Cole ST (1991) Molecular genetics and pathogenesis of *Clostridium perfringens*. Microbiol Rev 55:621-648

Ryu S, Labbe RG (1989) Coat and enterotoxin-related proteins in *Clostridium perfringens* spores. J Gen Microbiol 135:3109-3118

Ryu S, Labbe RG (1993) A 48 kilodalton enterotoxin-related protein from *Clostridium perfringens* vegetative and sporulating cells. Int J Food Microbiol 17:343-348

Saito M (1990) Production of enterotoxin by *Clostridium perfringens* derived from humans, animals, foods, and the natural environment in Japan. J Food Prot 53:115-118

Saito M, Matsumoto M, Funabashi M (1992) Detection of *Clostridium perfringens* enterotoxin gene by the polymerase chain reaction amplification procedure. Int J Food Microbiol 17:47-55

Salyers AA, Whitt, DD (1994) Bacterial pathogenesis: A Molecular Approach. Washington DC: ASM Press

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Seymour TA, Preston JF, Wicker L, Lindsay JA, Marshall M (1991) Purification and properties of pectinesterases of Marsh White grapefruit pulp. J Agri Food Chem 39:1080-1085

Shandera WX, Tacket CO, Blake PA (1983) Food poisoning due to *C. perfringens* in the United States. J Infec Dis 147:167-170

Skjelkvale R, Uemura T (1977) Experimental diarrhoea in human volunteers following oral admistration of *Clostridium perfringens* enterotoxin. J Appl Bacteriol 43:281-286

Smith LD, Gardner MV (1949) The occurrence of vegetative cells of *Clostridium perfringens* in soil. J Bacteriol 58:407-408

Stelma GN, Crawford RG, Spaulding PL, Twedt RM (1985) Evidence that *Clostridium perfringens* produces only one enterotoxin. J Food Prot 48:232-233

Stewart GSAB, Lubinsky-Mink S, Jackson CG, Cassel A, Kuhn J (1986) pHG165: a pBR322 copy number derivative of pUC18 for cloning and expression. Plasmid 15:172-181

Strauch MA, Hoch JA (1993) Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. Mol Microbiol 7:337-342

Stringer MF (1985) *Clostridium perfringens* type A food poisoning. In: Boriello SP (ed) Clostridia in Gastrointestinal Disease. Boca Raton, FL: CRC Press, pp 117-143

Sutton RGA, Hobbs BC (1965) Food poisoning caused by heat-sensitive *Clostridium welchii*. A report of five recent outbreaks. J Hyg Camb 66:135-146

Taylor AW, Gordon WS (1940) A survey of the types of *Cl. welchii* present in soil and in the intestinal contents of animals and man. J Pathol Bacteriol 50:271-277

Todd ECD (1989) Preliminary estimates of costs of foodborne disease in the United States. J Food Prot 52:595-601

Van Damme-Jongsten M, Rodhouse J, Gilbert RJ, Notermans S (1990) Synthetic DNA probes for detection of enterotoxigenic *Clostridium perfringens* strains isolated from outbreaks of food poisoning. J Clin Microbiol 28:131-133

Van Damme-Jongsten M, Wernars K, Notermans S (1989) Cloning and sequencing of the *Clostridium perfringens* enterotoxin gene. Anton van Leeuw 56:181-190

Van Der Kelen D, Lindsay JA (1991) Isolation, purification and partial characterization of a new extracellular cytotoxin from a virulent clinical strain of *Listeria monocytogenes* serotype 4b, and an avirulent, non hemolytic variant ATCC 15313 serotype 1/2a. J Food Safety 11:81-98

Whitaker JR, Granum PE (1980) The role of amino groups in the biological and antigenic activities of *Clostridium perfringens* type A enterotoxin. J Food Biochem 4:201-217

Wieckowski EU, Wnek AP, McClane BA (1994) Evidence that an ~ 50-kDa mammalian plasma membrane protein with receptor-like properties mediates the amphiphilicity of specifically bound *Clostridium perfringens* enterotoxin. J Biol Chem 269:10838-10848

Wnek AP, McClane BA (1986) Comparison of receptors for *Clostridium perfringens* type A and cholera enterotoxins in isolated rabbit intestinal brush border membranes. Microbial Path 1:89-100

Wnek AP, McClane BA (1989) Preliminary evidence that *Clostridium perfringens* type A enterotoxin is present in a 160,000-M_r Complex in mammalian membranes. Infect and Immun 57:574-581.

BIOGRAPHICAL SKETCH

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